

## WEST Search History





DATE: Tuesday, September 07, 2004

Hide?	Set Name	Query	Hit Count
		<i>DB=USPT; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L1	c-terminal.clm. and n-terminal.clm.	651
<input type="checkbox"/>	L2	L1 and heavy.clm. and light.clm.	16
<input type="checkbox"/>	L3	('6461617')!.PN.	1
<input type="checkbox"/>	L4	(cell or receptor or binding).clm. same translocat\$.clm.	164
<input type="checkbox"/>	L5	L4 and (botulin or botulinum or botoxin or clostridia or clostridium or clostridial or neurotoxin or neuro-toxin)	19
		<i>DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L6	hybrid.ti. and \$toxin.ti.	51
		<i>DB=USPT; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L7	US-6444209-B1.did.	1
<input type="checkbox"/>	L8	US-6444209-B1.did.	1
		<i>DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L9	recombinant\$.ti,ab,clm. and botul\$.ti,ab,clm.	106
<input type="checkbox"/>	L10	L9 not l2 not l5 not l6	101
<input type="checkbox"/>	L11	L10 not l4	101
		<i>DB=DWPI; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L12	2004028460	3
		<i>DB=EPAB; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L13	WO-2004028460-A2.did.	0
		<i>DB=JPAB,DWPI; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L14	2004028460	3
		<i>DB=EPAB,JPAB,DWPI; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L15	2004028460	3
		<i>DB=USPT,PGPB,JPAB,EPAB; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L16	(WO-2004028460-A2)! [pn]	0
		<i>DB=EPAB,JPAB,DWPI; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L17	028460	0
<input type="checkbox"/>	L18	2004028460	3
<input type="checkbox"/>	L19	probiotic.ti. and composition.ti. and lactic.ti. and acid.ti.	8
		<i>DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L20	williams.in. and botulin\$	127

☐ L21 williams.in. and james!.in. and botulin\$

27

END OF SEARCH HISTORY

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Search Results - Record(s) 1 through 1 of 1 returned.

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L3: Entry 1 of 1

File: USPT

Oct 8, 2002

US-PAT-NO: 6461617

DOCUMENT-IDENTIFIER: US 6461617 B1

**\*\* See image for Certificate of Correction \*\***

TITLE: Recombinant toxin fragments

DATE-ISSUED: October 8, 2002

US-CL-CURRENT: 424/236.1; 424/157.1, 424/164.1, 424/167.1, 424/178.1, 424/179.1,  
424/184.1, 424/234.1, 424/235.1, 424/239.1, 424/247.1, 435/252.33, 435/69.1,  
435/69.7, 435/70.1, 435/71.1, 435/71.2, 530/300, 530/350, 530/825, 536/23.4,  
536/23.7

INT-CL: [07] A61 K 39/02, A61 K 39/38, A61 K 39/00, C12 P 21/06, C12 P 21/04

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- ☐ 1. [6787517](#). 29 Dec 00; 07 Sep 04. Agent and methods for treating pain. Gil; Daniel W., et al. 514/1; 514/14 514/2. A01N061/00 A01N037/18 B61K031/00 B61K038/00 B61K038/28.
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- ☐ 2. [6776990](#). 08 Apr 99; 17 Aug 04. Methods and compositions for the treatment of pancreatitis. Sachs; George, et al. 424/192.1; 424/193.1. A61K039/00.
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- ☐ 3. [6682744](#). 09 Aug 00; 27 Jan 04. Pro-gut maturation and anti-inflammatory effects of lactobacillus and lactobacillus secreted proteins, carbohydrates and lipids. Panigrahi; Pinaki. 424/234.1; 424/184.1 424/262.1 424/93.45 514/2 530/350 530/825. A61K039/02 A61K039/00 A61K038/00 A01N063/00 C07K001/00.
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- ☐ 4. [6632440](#). 29 May 01; 14 Oct 03. Methods and compounds for the treatment of mucus hypersecretion. Quinn; Conrad Padraig, et al. 424/239.1; 424/236.1 424/282.1 424/434 424/810 435/325 435/368 435/371 435/6 435/69.1 435/7.1 514/12 514/14 514/2 530/350. A61K039/68 A61K039/00 C07K014/00.
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- ☐ 5. [6506399](#). 04 Oct 01; 14 Jan 03. Biodegradable botulinum toxin implant. Donovan; Stephen. 424/423; 424/184.1 424/236.1 424/422 424/426. A61K039/02 A61K039/00.
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- ☐ 6. [6461617](#). 23 Feb 99; 08 Oct 02. Recombinant toxin fragments. Shone; Clifford Charles, et al. 424/236.1; 424/157.1 424/164.1 424/167.1 424/178.1 424/179.1 424/184.1 424/234.1 424/235.1 424/239.1 424/247.1 435/252.33 435/69.1 435/69.7 435/70.1 435/71.1 435/71.2 530/300 530/350 530/825 536/23.4 536/23.7. A61K039/02 A61K039/38 A61K039/00 C12P021/06 C12P021/04.
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- ☐ 7. [6451593](#). 12 May 99; 17 Sep 02. Design principle for construction of expression constructs for gene therapy. Wittig; Burghardt, et al. 435/320.1; 435/325 435/455 435/69.1 514/44 536/23.1 536/23.5. C12N015/00 C12N005/00 C12P021/06 C07H021/00 A01N043/04.
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- ☐ 8. [6416959](#). 25 Feb 00; 09 Jul 02. System for cell-based screening. Giuliano; Kenneth, et al. 435/7.2; 250/201.3 348/345 356/300 356/326 356/328 382/141 382/255 435/288.3 435/288.4 435/29 435/40.5 435/40.51 435/7.21 436/172 436/546 436/800 436/809. G01N033/533.
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- ☐ 9. [6312708](#). 21 Jul 00; 06 Nov 01. Botulinum toxin implant. Donovan; Stephen. 424/423; 424/184.1 424/236.1 424/422 424/426. A61K039/02 A61K039/00.
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- ☐ 10. [6197755](#). 27 May 99; 06 Mar 01. Compositions and methods for delivery of genetic material. Carrano; Richard A., et al. 514/44; 424/278.1. A61K031/7088 A61K045/05.
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- ☐ 11. [6100056](#). 24 Dec 96; 08 Aug 00. Nisins. Gasson; Michael John, et al. 435/69.1; 435/252.3 435/320.1 435/471. C12N001/21 C12N015/31 C12N015/74.
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- ☐ 12. [6022950](#). 07 Jun 95; 08 Feb 00. Hybrid molecules having translocation region and cell-binding region. Murphy; John R.. 530/350; 530/351 530/387.3 530/388.1. C07K014/00 C07K014/485 C07K014/55 C07K016/28.
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- ☐ 13. [5981505](#). 26 Nov 97; 09 Nov 99. Compositions and methods for delivery of genetic material.

Weiner; David B., et al. 514/44; 424/278.1 514/615 514/818. A61K045/05 A61K048/00 A61K031/00.

☐ 14. 5965406. 07 Jun 95; 12 Oct 99. Recombinant DNAs encoding three-part hybrid proteins. Murphy; John R.. 435/69.7; 435/252.33 435/320.1 536/23.4. C12N001/21 C12N015/12 C12N015/63 C12P021/02.

☐ 15. 5962428. 16 Sep 96; 05 Oct 99. Compositions and methods for delivery of genetic material. Carrano; Richard A., et al. 514/44; 424/278.1. A61K031/70 A61K045/05.

☐ 16. 5817637. 13 Jan 97; 06 Oct 98. Genetic immunization. Weiner; David B., et al. 514/44; 424/278.1 435/975 514/615 514/818. A61K045/05 A61K048/00 A61K031/00.

☐ 17. 5763190. 21 Sep 94; 09 Jun 98. Methods for the identification of compounds capable of inducing the nuclear translocation of a receptor complex comprising the glucocorticoid receptor type II and viral protein R interacting protein. Weiner; David B., et al. 435/7.1; 435/69.1 435/7.8. G01N033/53 C12P021/06 C12N015/00.

☐ 18. 5677274. 25 Jun 93; 14 Oct 97. Anthrax toxin fusion proteins and related methods. Leppla; Stephen H., et al. 514/2;. A61K039/00.

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Terms	Documents
L4 and (botulin or botulinum or botoxin or clostridia or clostridium or clostridial or neurotoxin or neuro-toxin)	19

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- ☐ 1. [20030113345](#). 19 Nov 02. 19 Jun 03. Hybrid lt-a/ct-b holotoxin for use as an adjuvant. Clements, John D. 424/203.1; 424/241.1 A61K039/116 A61K039/108.
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- ☐ 2. [6204246](#). 31 Dec 97; 20 Mar 01. Hybrid toxin. Bosch; Hendrik Jan, et al. 514/12; 514/2 530/350 530/825. A01N037/18 C07K014/325.
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- ☐ 3. [6019973](#). 05 Jan 98; 01 Feb 00. Hybrid molecules between heat-labile enterotoxin and cholera toxin B subunits. Holmgren; Jan, et al. 424/185.1; 424/184.1 424/236.1 424/241.1 424/261.1 530/350. A61K039/00 A61K039/02 A61K039/108 A61K039/106.
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- ☐ 4. [5780024](#). 21 Jun 96; 14 Jul 98. Superoxide dismutase/tetanus toxin fragment C hybrid protein. Brown; Robert H., et al. 424/94.4; 435/189. A61K038/44 C12N015/00 C12N009/02.
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- ☐ 5. [5736131](#). 21 Feb 96; 07 Apr 98. Hybrid toxin. Bosch; Hendrik Jan, et al. 800/300; 424/93.1 424/93.2 424/93.461 435/252.3 435/252.31 435/254.11 435/320.1 435/69.7 514/12 514/2 530/350 536/23.4 536/23.71. A01N063/00 A01N037/18 C07K014/325 C12P021/02.
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- ☐ 6. [5110905](#). 27 Jul 90; 05 May 92. Activated *Bacillus thuringiensis* delta-endotoxin produced by an engineered hybrid gene. Witt; Daniel P., et al. 530/350; 435/69.1 435/71.1. C07K003/00 C12P021/06 C12P021/04.
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- ☐ 7. [4468382](#). 15 Jul 82; 28 Aug 84. Polypeptide-toxin hybrid protein. Bacha; Patricia, et al. 514/19; 436/501 530/323 530/345. A61K037/00 C07C103/52.
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- ☐ 8. [JP402072872A](#). 28 Apr 89. 13 Mar 90. NEW HYBRID INSECTICIDAL TOXIN. WILCOX, EDWARD, et al. C12N009/00; A01N063/00 C07K013/00 C07K015/12 C12N001/21 C12N015/62 C12N015/31 C12P021/02.
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- ☐ 9. [WO009634893A1](#). 02 May 96. 07 Nov 96. HYBRID MOLECULES BETWEEN HEAT-LABILE ENTEROTOXIN AND CHOLERA TOXIN B SUBUNITS. HOLMGREN, JAN, et al. C07K019/00; C07K014/28 C07K014/245 C12N015/62 A61K039/116.
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- ☐ 10. [WO009506730A1](#). 01 Sep 94. 09 Mar 95. HYBRID TOXIN. BOSCH, HENDRIK JAN, et al. C12N015/32; C07K014/325 C12N015/82 A01H005/00 A01N063/00.
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- ☐ 11. [WO009316186A1](#). 12 Feb 93. 19 Aug 93. HYBRID DNA THAT CODES FOR A SUBUNIT OF A BACTERIAL TOXIN WITH A HEMOLYSIN. BRAHMBHATT, HIMANSHU, et al. A61K039/02; C07K007/00 C07K007/10 C12N015/62 C12N015/74.
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- ☐ 12. [EP000206613A2](#). 09 Jun 86. 30 Dec 86. Activated *bacillus thuringiensis* delta-endotoxin produced by an engineered hybrid gene.. WITT, DANIEL P, et al. 435/69.1 435/252.31. C07K013/00; C12N001/20 C12N015/00 C12P021/02.
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- ☐ 13. [US 6713063B](#). New modified, insecticidal *Bacillus thuringiensis* hybrid crystal protein comprises domains of delta-endotoxin Cry1A and Cry1F proteins, useful as an insecticide for coleopteran, dipteran or lepidopteran insects. MALVAR, T, et al. A01N037/18 A61K039/00

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☐ 14. WO2004021992A. Administering a therapeutic molecule to a subject comprises providing a hybrid protein comprising the therapeutic molecule and tetanus toxin fragment C and administering the hybrid protein into the brain or spinal cord parenchyma. BROWN, R H, et al. A61K000/00.

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☐ 15. EP 1386927A. New hybrid compounds comprising a Shiga Toxin B subunit, useful as a contrast agent for the in vivo diagnosis of tumors (e.g. intestinal tumor) expressing Gb3 receptor, or as a drug for killing tumor cells over-expressing Gb3 receptor. FLORENT, J, et al. A61B000/00 A61K047/48 C07K014/25.

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☐ 16. US 6444209B. New hybrid botulinal neurotoxin, useful to treat dystonia, comprises light and heavy chain not of the same serotype and linked by reducible disulfide homobifunctional linker. BRADSHAW, M, et al. A61K039/08 A61K039/385 C12N009/52.

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☐ 17. WO 200215701A. *Bacillus thuringiensis* hybrid toxin H04 for controlling insects, e.g. fall army worm or European cornborer, and for creating insect resistant plants, comprises domains I and II of Cry1Ab and domain III of Cry1C. CAROZZI, N B, et al. A01H005/00 A01H005/10 A01N063/00 A01N063/02 C07K014/325 C07K019/00 C12N005/10 C12N015/09 C12N015/32 C12N015/62 C12N015/82 C12P021/02.

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☐ 18. WO 200189456A. New hybrid heat labile enterotoxin of *Escherichia coli* and the B-subunit of the cholera enterotoxin of *Vibrio cholerae*, useful as adjuvants for vaccines, particularly for stimulating antibodies or cell mediated immune responses. CLEMENTS, J D. A61K000/00 A61K039/108 A61K039/116.

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☐ 19. US20010014469A. Novel hybrid pesticidal toxin useful as insecticide, comprises cytotoxic agent, preferably diphtheria toxin and pest gut epithelial cell recognition portion of protein linked by a peptide linker. CULVER, P, et al. C12N009/00.

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☐ 20. US20010049142A. A kit comprising endotoxin free dyes for the labelling and preparation of hybrid cells useful for the treatment of disorders including cancer. WAGNER, T, et al. A61K035/14 A61K038/00 A61K039/00 A61K048/00 A61P003/10 A61P031/00 A61P031/04 A61P031/10 A61P033/00 A61P033/02 A61P033/04 A61P033/10 A61P035/00 A61P037/00 A61P037/02 A61P037/04 A61P037/06 A61P043/00 C12N005/00 C12N005/06 C12N005/10 C12N005/12 C12N005/22 C12N005/24 C12N015/02 A61K038:20 A61K039/00.

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☐ 21. US 6222104B. Novel transgenic maize seed for hybrid maize plant production, comprising expression cassette linked operably with CAMV 35S promoter, alcohol dehydrogenase intron, insecticidal Cry1Ab toxin gene or NOS terminator. KRIER, M, et al. A01H001/00 A01H005/00 A01H005/10.

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☐ 22. WO 200114562A. Novel hybrid insecticidal toxin useful for controlling insects such as *Spodoptera exigua* and *Plutella xylostella*, comprises domains I and II from Cry1F or Cry1B toxin joined to domain III from Cry1C toxin. BOSCH, H J, et al. A01H005/00 A01N063/02 C07K014/325 C12N015/32 C12N015/62 C12P021/02.

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☐ 23. US 5939070A. Hybrid botulinal neurotoxin comprising botulinal light and heavy chains of different serotypes. BRADSHAW, M, et al. A61K039/08 A61K039/385 C12N009/52 C12P021/06.

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☐ 24. US 5830478A. Delivery of functional domains of diphtheria toxin to cellular targets -

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comprises administration of a hybrid reagent and a second reagent, each having an endosomally active domain. RASO, V A, et al. A61K039/395.

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☐ 25. US 6392123B. Generation of conditionally female sterile plants - by expressing enzyme converting protoxin to toxin, under control of female preferential promoter in female plant tissue, useful for hybrid seed production. CROSSLAND, L D, et al. A01H005/00 A01H005/10 C12N005/10 C12N015/09 C12N015/29 C12N015/82.

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☐ 26. US 5780024A. New hybrid protein of superoxide dismutase and tetanus toxin fragment C - having increased uptake by neurons and retention of enzymatic activity in these cells, for treating neurological diseases associated with oxidative stress. BROWN, R H, et al. A61K038/44 C12N009/02 C12N015/00.

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☐ 27. FR 2754543A. Toxin-defective Bordetella strain for use in vaccines, etc. - expressing hybrid of filamentous haemagglutinin and heterologous protein. CAPRON, A, et al. A01N063/00 A61K009/127 A61K038/16 A61K039/00 A61K039/02 A61K039/10 A61P011/00 A61P031/04 A61P037/04 C07K014/235 C07K019/00 C12N001/21 C12N015/09 C12N001/21 C12R001:01.

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☐ 28. RU 2096458C. A hybrid receptor toxin S 304 and an DNA fragment NS 304 coding it - is a potential HIV therapeutic agent. SIDOROV, A V, et al. C12N015/12 C12N015/31.

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☐ 29. RU 2077585C. Recombinant plasmid pDTI 23 - encodes hybrid protein comprising diphtheria toxin and interleukin 2. ANISIMOVA, V A, et al. C12N015/26 C12N015/31.

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☐ 30. WO 9713410A. Hybrid molecule for targeting compound, especially a toxin, into cells - includes polypeptide able to transport the compound across cytoplasmic membranes and amidated ligand, useful for treatment of cancer. FISHER, C E, et al. A01N037/18 A01N043/50 C07H021/04 C12P021/04 C12P021/06 G01N033/48.

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☐ 31. US 6019973A. Heat-labile enterotoxin B sub-unit and cholera toxin B sub-unit hybrid protein - opt. fused to immunogenic sequence for use in vaccines against enterotoxin-induced illness. HOLMGREN, J, et al. A61K039/00 A61K039/02 A61K039/106 A61K039/108 A61K039/116 C07K014/24 C07K014/245 C07K014/28 C07K019/00 C12N015/09 C12N015/62 C12P021/02.

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☐ 32. RU 2065495C. Hybrid strain of *Saccharomyces cerevisiae*- *Saccharomyces bayanus* used in wine making - has ability to synthesise 'killer' toxin lysing sensitive, closely related yeast cells. KARDASH, N K, et al. C12G001/02 C12N001/16 C12N001/16 C12R001:85.

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☐ 33. US 5736131A. New *Bacillus thuringiensis* hybrid toxin fragment - derived from two different cry proteins, and related hybrid toxins, recombinant DNA, vectors, transformed plants and microorganisms, for insect control.. BOSCH, H J, et al. A01H005/00 A01M001/20 A01N000/00 A01N037/18 A01N037/46 A01N063/00 A01N063/02 C07H000/00 C07K014/325 C07K019/00 C12N015/09 C12N015/32 C12N015/62 C12N015/82 C12P021/02.

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☐ 34. WO 9322450A. New chimeric diphtheria toxin contg. antibody complementarity determining region - in loop region of binding domain, for killing antibody reactive cells, also as hybrid, for targetted delivery of protein or nucleic acid. CHOE, S, et al. C07K003/00 C07K013/00 C07K015/00 C12P021/08.

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☐ 35. DE 4113385A. New hybrid transposon - contain pertussis toxin operon and derived plasmids for expressing the toxin, esp. in *Bordetella bronchiseptica*, useful for whooping cough vaccine prodn..

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☐ 36. US 5152980A. Induction of tolerance to foreign antigen - by admin. of IL-2-toxin hybrid or anti-IL-2-receptor antibody. KELLEY, V E, et al. A61K037/02 A61K039/00.

☐ 37. SU 1742325A. Novel strain of hybrid cultivated *Mus musculus* L. cells - is used as producer of monoclonal antibodies to cholera enterotoxin of Eltor bio-type. KIRILLOVA, E F, et al. A61K039/395 C12N005/00.

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"PATNO\_JP402079970A" 42. JP 02079970A. Hybrid monoclonal antibody having double specificity - where one corresponds to human lymphotoxin and other specificity corresponds to target antigen. A61K039/39 C07K015/12 C12N005/16 C12P021/08 C12R001/91.

☐ 43. EP 338497A. Polydroma producing bi:specific hybrid monoclonal antibody - with specificity for human lymphotoxin and marker, esp. horseradish and assay of lymphotoxin. IWASA, S, et al. A61K039/39 C12N005/00 C12N009/00 C12N015/00 C12P021/00 C12R001/91 G01N033/57.

☐ 44. EP 331470A. New hybrid *Bacillus thuringiensis* gene - deriv from Kurstaki and Berliner strains, used to produce toxin active against Lepidoptera insects. GILROY, T E. A01N063/00 C07H015/12 C07K013/00 C12N001/20 C12N005/10 C12N015/00 C12N015/09 C12N015/32 C12N015/63 C12P021/00 C12R001/07.

☐ 45. EP 325400A. Hybrid *Bacillus thuringiensis* gene for control of Lepidopteran insects - contg. part of var. kurstaki HD-73 and HD-1 toxin genes. GILROY, T E, et al. A01N063/00 A01N063/02 C07H021/04 C07K013/00 C07K014/325 C12N001/15 C12N001/19 C12N001/20 C12N001/21 C12N005/00 C12N015/00 C12N015/09 C12N015/32 C12R001/38 C12N001/21 C12R001:39.

☐ 46. CA 1217156A. New fusion protein prepn. contg. active fragment of diphtheria toxin - coupled to cell specific ligand, by expressing new hybrid gene, useful as specific cytotoxic agent. MURPHY, J R. C07K007/10 C07K013/00 C07K015/00 C12N015/00 C12P021/02.

☐ 47. DE 3686452G. *Bacillus thuringiensis kurstaki* delta-endotoxin - prepd. using a hybrid gene which is cloned into a plasmid which is transformed into a prokaryotic host. ANILIONIS, A, et al. C07K003/00 C07K013/00 C07K015/12 C12N001/20 C12N015/32 C12P021/02 C12P021/06 C12N001/20 C12R001:19.

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S2	58675	BOTULIN? OR BOTULINUM? OR BOTULISM? OR BOTOX?
S3	4812	S1 AND S2
S4	3187	S3/1996:2004
S5	1625	S3 NOT S4
S6	888	RD (unique items)
S7	161	S6 AND (HEAVY? OR LARGE? OR H1 OR H1A OR H) AND (LIGHT? OR SMALL? OR L)
S8	189	S6 AND CHAIN?
S9	13	(S7 OR S7) AND RECOMBINANT?
S10	1847	S1 (15N) S2
S11	1137	S10/1996:2004
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L11: Entry 53 of 101

File: USPT

Dec 17, 2002

DOCUMENT-IDENTIFIER: US 6495143 B2

TITLE: Botulinum neurotoxin vaccineAbstract Text (1):

Using the nontoxic heavy chain fragment from botulinum neurotoxins A-G, compositions and methods of use in inducing an immune response which is protective against intoxication with botulinum in subjects is described.

## CLAIMS:

1. A recombinant DNA construct comprising: (i) a vector VEE, and (ii) at least one nucleic acid fragment comprising a carboxy terminal heavy chain fragment from any of BoNTA, BoNTB, BoNTC, BoNTD, BoNTE, BoNTF, and BoNTG.
2. A recombinant DNA construct according to claim 1 wherein said vector is an expression vector.
3. A recombinant DNA construct according to claim 1 wherein said vector further comprises a prokaryotic vector.
4. A recombinant DNA construct according to claim 1 wherein said vector further comprises another eukaryotic vector.
5. The recombinant DNA construct according to claim 1 wherein said construct is p3014-40A.
6. The recombinant DNA construct according to claim 1 wherein said construct is p3014-114a1.
7. The recombinant DNA construct according to claim 1 wherein said construct is p3014-102a1.
8. The recombinant DNA construct according to claim 1 wherein said construct is p3014-73B.
9. The recombinant DNA construct according to claim 1 wherein said construct is p3014-110C.
10. The recombinant DNA construct according to claim 1 wherein said construct is p3014-75E.
11. The recombinant DNA construct according to claim 1 wherein said construct is p3014-77F.
12. The recombinant DNA construct according to claim 1 wherein said construct is p3014-107G.
13. A host cell transformed with a recombinant DNA construct according to claim 1.

14. A host cell transformed with a recombinant DNA construct according to claim 3.
15. A host cell transformed with a recombinant DNA construct according to claim 4.

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- 
- ☐ 1. [20040142455](#). 05 Dec 03. 22 Jul 04. Recombinant botulinum toxins having a soluble C-terminal portion of a heavy chain, an N-terminal portion of a heavy chain and a light chain. Williams, James A.. 435/252.33; 435/254.2 435/320.1 435/348 435/69.3 530/350 536/23.7 C12P021/02 C12N001/21 C12N001/18 C12N005/06.
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- ☐ 2. [20040115215](#). 05 Dec 03. 17 Jun 04. Recombinant botulinum toxins with a soluble C-terminal portion, an N-terminal portion and a light chain. Williams, James A.. 424/184.1; A61K039/395 A61K039/00 A61K039/38.
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- ☐ 4. [20030219457](#). 15 Oct 02. 27 Nov 03. Soluble recombinant botulinum toxins. Williams, James A.. 424/199.1; 424/186.1 424/234.1 435/6 C12Q001/68 A61K039/12 A61K039/02.
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- ☐ 5. [20030215468](#). 30 Jan 03. 20 Nov 03. Soluble recombinant botulinum toxin proteins. Williams, James A., et al. 424/239.1; 435/252.3 435/70.21 530/388.4 A61K039/08 C12P021/04 C12N001/21 C07K016/12.
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- ☐ 6. [20030211096](#). 02 Aug 02. 13 Nov 03. Compositions and methods for the treatment of tumor. Ashkenazi, Avi J., et al. 424/130.1; 435/320.1 435/326 435/69.1 530/388.1 536/23.53 A61K039/395 C07H021/04 C12P021/02 C12N005/06 C07K016/40.
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- ☐ 7. [20030175900](#). 02 Aug 02. 18 Sep 03. Compositions and methods for the treatment of tumor. Ashkenazi, Avi J., et al. 435/71.2; 424/130.1 435/320.1 435/326 435/70.21 530/388.1 536/23.53 C12P021/04 C07H021/04 A61K039/395 C12P021/02 C12N005/06 C07K016/40.
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- ☐ 9. [20030054493](#). 16 Nov 01. 20 Mar 03. Identification of neutralizing epitopes of toxin A and toxin B for the treatment of C. difficile disease. Williams, James A., et al. 435/69.3; 424/247.1 435/252.33 435/320.1 C12P021/02 C12N001/21 A61K039/08 C12N015/74.
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- ☐ 10. [20020012658](#). 16 Jun 99. 31 Jan 02. PREVENTION AND TREATMENT OF VEROTOXIN-INDUCED DISEASE. WILLIAMS, JAMES A., et al. 424/93.2; A61K048/00 A01N063/00.
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- ☐ 11. [6652857](#). 16 Jun 99; 25 Nov 03. Methods for producing avian verotoxin antitoxin. Williams, James A., et al. 424/169.1; 424/130.1 424/150.1 436/547. A61K039/40 A61K039/395 G01N033/53.
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- ☐ 12. [6613329](#). 26 May 98; 02 Sep 03. Vaccine and antitoxin for treatment and prevention of C. difficile disease. Kink; John A., et al. 424/164.1; 424/184.1 530/387.1. A61K039/40 A61K039/00 C07K016/00.
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- ☐ 13. [6573003](#). 16 Nov 01; 03 Jun 03. Identification of neutralizing epitopes of toxin A and toxin B

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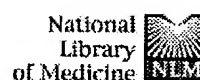
☐ 27. WO009808540A1. 28 Aug 97. 05 Mar 98. MULTIVALENT VACCINE FOR CLOSTRIDIUM BOTULINUM NEUROTOXIN. WILLIAMS, JAMES A, et al. A61K039/00; A61K039/38 A61K038/08 C12P021/06 C12P021/04 C12P021/08 C12N015/00 C12N015/09 C12N015/63 C12N015/70 C12N015/74 C07K016/00.

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**Nucleotide sequence of the gene coding for *Cl* botulinum (*Clostridium argentinense*) type *G* ne genealogical comparison with other clostridial neurotoxins.**

Campbell K, Collins MD, East AK.

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Department of Microbiology, Institute of Food Research  
Laboratory, Earley Gate, UK.

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The neurotoxin gene from *Clostridium botulinum* type *G* as a series of overlapping DNA fragments generated us polymerase chain reaction (PCR) technology and primers conserved regions of published botulinal toxin (BoNT) s The 5'-end of the gene was obtained using a primer bas conserved region of the nontoxic-nonhaemagglutinin gen upstream of the toxin gene. Translation of the nucleotid derived from the cloned PCR fragments demonstrated t gene encodes a protein of 1297 amino acid residues (rm Comparative alignment of the determined BoNT/*G* sequ those of other clostridial neurotoxins revealed highest relatedness (approx. 58% amino acid identity) with BoN proteolytic and non-proteolytic *C. botulinum*. Tetanus to

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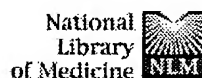
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**Nucleotide sequence of the gene coding for Cl  
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genealogical comparison with other clostridial  
neurotoxins.**

**Campbell K, Collins MD, East AK.**

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Department of Microbiology, Institute of Food Research  
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The neurotoxin gene from *Clostridium botulinum* type G as a series of overlapping DNA fragments generated us polymerase chain reaction (PCR) technology and primers conserved regions of published botulinal toxin (BoNT) s The 5'-end of the gene was obtained using a primer bas conserved region of the nontoxic-nonhaemagglutinin gen upstream of the toxin gene. Translation of the nucleotid derived from the cloned PCR fragments demonstrated t gene encodes a protein of 1297 amino acid residues (rm Comparative alignment of the determined BoNT/G sequ those of other clostridial neurotoxins revealed highest relatedness (approx. 58% amino acid identity) with BoN proteolytic and non-proteolytic *C. botulinum*. Tetanus to

and other BoNT types revealed lower levels of relatedn  
BoNT/G (approximate range 35-42% amino acid identity

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**Molecular characterization of the clusters of encoding the botulinum neurotoxin complex in *Clostridium argentinense* type G and nonproteolytic *Clostridium botulinum* type B.**

**Bhandari M, Campbell KD, Collins MD, East AK.**

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Department of Microbiology, Institute of Food Research  
Gate, Whiteknights Road, Reading, RG6 6BZ, UK.

Related  
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The cluster of genes encoding components of the proteolytic botulinum neurotoxin complex has been mapped and cloned in *Clostridium botulinum* type G strain ATCC 27322. Determination of the nucleotide sequence of the region has revealed open reading frames encoding nontoxic components of the complex, including the gene encoding BoNT/G (botG). The arrangement of botG differs from that in strains of other antigenic toxin types. Immediately upstream of botG lies a gene encoding a protein of 1198 amino acids, which shows homology with the nontoxic-nonhemagglutinin (NTNH) component of the proteolytic complex. Further upstream there are genes encoding proteins with homology to hemagglutinin components (HA-17, HA-70) and a putative positive regulator of gene expression (P-21). Sequence comparison has shown that BoNT/G has highest homology

BoNT/B. The sequence of the BoNT-cluster of genes in non-proteolytic *C. botulinum* type B strain Eklund 17B has extended to include the complete NTN<sub>H</sub> and HA-17, an HA-70 gene sequences. Comparison of NTN<sub>H</sub>/G with ot reveals that it shows highest homology with NTN<sub>H</sub>/B<sub>c</sub> with the genealogical affinity shown between BoNT/G<sub>a</sub> genes.

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E2	1		BOTULINUM ANTITOXIN --TOXICITY --TO
E3	0		*BOTULINUM TOXIN
E4	1502	4	BOTULINUM TOXIN TYPE A
E5	536		BOTULINUM TOXIN TYPE A --ADMINISTRATION AND DO
E6	221		BOTULINUM TOXIN TYPE A --ADVERSE EFFECTS --AE
E7	15		BOTULINUM TOXIN TYPE A --ANALYSIS --AN
E8	21		BOTULINUM TOXIN TYPE A --ANTAGONISTS AND INHIB
E9	8		BOTULINUM TOXIN TYPE A --BIOSYNTHESIS --BI
E10	5		BOTULINUM TOXIN TYPE A --BLOOD --BL
E11	3		BOTULINUM TOXIN TYPE A --CHEMICAL SYNTHESIS --
E12	50		BOTULINUM TOXIN TYPE A --CHEMISTRY --CH

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E13	1	BOTULINUM TOXIN TYPE A --CLASSIFICATION --CL
E14	11	BOTULINUM TOXIN TYPE A --CONTRAINDICATIONS --C
E15	13	BOTULINUM TOXIN TYPE A --ECONOMICS --EC
E16	25	BOTULINUM TOXIN TYPE A --GENETICS --GE
E17	12	BOTULINUM TOXIN TYPE A --HISTORY --HI
E18	53	BOTULINUM TOXIN TYPE A --IMMUNOLOGY --IM
E19	21	BOTULINUM TOXIN TYPE A --ISOLATION AND PURIFIC
E20	49	BOTULINUM TOXIN TYPE A --METABOLISM --ME
E21	20	BOTULINUM TOXIN TYPE A --PHARMACOKINETICS --PK
E22	218	BOTULINUM TOXIN TYPE A --PHARMACOLOGY --PD
E23	5	BOTULINUM TOXIN TYPE A --POISONING --PO
E24	4	BOTULINUM TOXIN TYPE A --STANDARDS --ST

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S1 25 'BOTULINUM TOXIN TYPE A --GENETICS --GE'

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Ref	Items	RT	Index-term
E25	1		BOTULINUM TOXIN TYPE A --SUPPLY AND DISTRIBUTI
E26	838		BOTULINUM TOXIN TYPE A --THERAPEUTIC USE --TU
E27	31		BOTULINUM TOXIN TYPE A --TOXICITY --TO
E28	1		BOTULINUM TOXIN TYPE A --URINE --UR
E29	170		BOTULINUM TOXIN TYPE B
E30	100		BOTULINUM TOXIN TYPE C
E31	22		BOTULINUM TOXIN TYPE D
E32	63		BOTULINUM TOXIN TYPE E
E33	27		BOTULINUM TOXIN TYPE F
E34	1		BOTULINUM TOXIN TYPE G
E35	4166	11	BOTULINUM TOXINS

E36 731 BOTULINUM TOXINS --ADMINISTRATION AND DOSAGE -

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S2 1 'BOTULINUM TOXIN TYPE G'

?e e35

Ref	Items	Type	RT	Index-term
R1	4166		11	*BOTULINUM TOXINS
R2	4166	X		DC=D24.185.926.123.179. (BOTULINUM TOXINS)
R3	4166	X		DC=D24.185.926.640.75. (BOTULINUM TOXINS)
R4	170	X	1	BOTULIN
R5	0	X	1	CLOSTRIDIUM BOTULINUM TOXINS
R6	2329	R	10	BOTULISM
R7	882	R	109	CHOLINERGIC AGENTS
R8	1888	R	6	CLOSTRIDIUM BOTULINUM
R9	545	B	29	ANTI-DYSKINESIA AGENTS
R10	12046	B	17	BACTERIAL TOXINS
R11	8982	B	15	NEUROTOXINS
R12	1502	N	4	BOTULINUM TOXIN TYPE A

?p

>>>Related terms display completed...

?e r4

Ref	Items	Type	RT	Index-term
R1	170		1	*BOTULIN
R2	4166	X	11	BOTULINUM TOXINS

?s r1:r2

S3 4273 R1:R2

?e r2

Ref	Items	Type	RT	Index-term
R1	4166		11	*BOTULINUM TOXINS
R2	4166	X		DC=D24.185.926.123.179. (BOTULINUM TOXINS)
R3	4166	X		DC=D24.185.926.640.75. (BOTULINUM TOXINS)
R4	170	X	1	BOTULIN
R5	0	X	1	CLOSTRIDIUM BOTULINUM TOXINS
R6	2329	R	10	BOTULISM
R7	882	R	109	CHOLINERGIC AGENTS
R8	1888	R	6	CLOSTRIDIUM BOTULINUM
R9	545	B	29	ANTI-DYSKINESIA AGENTS
R10	12046	B	17	BACTERIAL TOXINS
R11	8982	B	15	NEUROTOXINS
R12	1502	N	4	BOTULINUM TOXIN TYPE A

?e r12

Ref	Items	Type	RT	Index-term
R1	1502		4	*BOTULINUM TOXIN TYPE A
R2	1502	X		DC=D24.185.926.123.179.50. (BOTULINUM TOXIN TYPE A)
R3	1502	X		DC=D24.185.926.640.75.50. (BOTULINUM TOXIN TYPE A)
R4	4166	B	11	BOTULINUM TOXINS
R5	1083	B	34	NEUROMUSCULAR AGENTS

?e botulinum toxins

Ref	Items	RT	Index-term
E1	27		BOTULINUM TOXIN TYPE F
E2	1		BOTULINUM TOXIN TYPE G
E3	4166	11	*BOTULINUM TOXINS
E4	731		BOTULINUM TOXINS --ADMINISTRATION AND DOSAGE -
E5	315		BOTULINUM TOXINS --ADVERSE EFFECTS --AE
E6	266		BOTULINUM TOXINS --ANALYSIS --AN
E7	46		BOTULINUM TOXINS --ANTAGONISTS AND INHIBITORS
E8	190		BOTULINUM TOXINS --BIOSYNTHESIS --BI
E9	69		BOTULINUM TOXINS --BLOOD --BL
E10	1		BOTULINUM TOXINS --CHEMICAL SYNTHESIS --CS
E11	169		BOTULINUM TOXINS --CHEMISTRY --CH
E12	39		BOTULINUM TOXINS --CLASSIFICATION --CL

Enter P or PAGE for more

?p

Ref	Items	Index-term
E13	9	BOTULINUM TOXINS --CONTRAINDICATIONS --CT
E14	2	BOTULINUM TOXINS --DIAGNOSTIC USE --DU
E15	20	BOTULINUM TOXINS --ECONOMICS --EC
E16	144	BOTULINUM TOXINS --GENETICS --GE
E17	18	BOTULINUM TOXINS --HISTORY --HI
E18	175	BOTULINUM TOXINS --IMMUNOLOGY --IM
E19	196	BOTULINUM TOXINS --ISOLATION AND PURIFICATION
E20	339	BOTULINUM TOXINS --METABOLISM --ME
E21	23	BOTULINUM TOXINS --PHARMACOKINETICS --PK
E22	853	BOTULINUM TOXINS --PHARMACOLOGY --PD
E23	3	BOTULINUM TOXINS --PHYSIOLOGY --PH
E24	38	BOTULINUM TOXINS --POISONING --PO

Enter P or PAGE for more

?s e16

S4 144 'BOTULINUM TOXINS --GENETICS --GE'

?p

Ref	Items	Index-term
E25	2	BOTULINUM TOXINS --RADIATION EFFECTS --RE
E26	2	BOTULINUM TOXINS --SECRETION --SE
E27	9	BOTULINUM TOXINS --STANDARDS --ST
E28	3	BOTULINUM TOXINS --SUPPLY AND DISTRIBUTION --S
E29	1281	BOTULINUM TOXINS --THERAPEUTIC USE --TU
E30	325	BOTULINUM TOXINS --TOXICITY --TO
E31	1	BOTULINUM TOXINS --URINE --UR
E32	1	BOTULINUMA
E33	1	BOTULINUMEXOTOXIN
E34	1	BOTULINUMFORGIFTNING
E35	1	BOTULINUMNEUROTOKSIN
E36	1	BOTULINUMTOKSIINI

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?p

Ref	Items	Index-term
E37	2	BOTULINUMTOKSIINILLA
E38	1	BOTULINUMTOKSIININ
E39	11	BOTULINUMTOKSIN
E40	3	BOTULINUMTOKSINBEHANDLING
E41	1	BOTULINUMTOXIM
E42	39	BOTULINUMTOXIN
E43	2	BOTULINUMTOXINBEHANDLUNG
E44	1	BOTULINUMTOXINPRAPARATEN
E45	1	BOTULINUMTOXINTHERAPIE
E46	2	BOTULINUN
E47	93	BOTULINUS
E48	1	BOTULINUSA

Enter P or PAGE for more

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Ref	Items	Index-term
E49	2	BOTULINUSINTOXIKATION
E50	13	BOTULINUSTOXIN

?s botuli?

S5 9156 BOTULI?

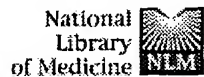
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Set	Items	Description
S1	25	'BOTULINUM TOXIN TYPE A --GENETICS --GE'
S2	1	'BOTULINUM TOXIN TYPE G'

S3	4273	R1:R2
S4	144	'BOTULINUM TOXINS --GENETICS --GE'
S5	9156	BOTULI?
?t	s2/9/all	

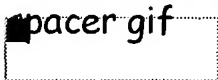
Set	Items	Description
S1	25	'BOTULINUM TOXIN TYPE A --GENETICS --GE'
S2	1	'BOTULINUM TOXIN TYPE G'
S3	4273	R1:R2
S4	144	'BOTULINUM TOXINS --GENETICS --GE'
S5	9156	BOTULI?
S6	9156	S1 OR S3 OR S4 OR S5
S7	4437	S6/1996:2004
S8	4719	S6 NOT S7
S9	576	S8 AND (RECOMBIN? OR CHIMER? OR MODIF? OR HYBRID? OR RECOM- BINED? OR GENE? OR MUTANT?)
S10	40	S9 AND LIGHT? AND HEAVY?
S11	0	S9 AND LC AND HC
S12	39	S10 AND CHAIN?
S13	22	S12 AND TERMIN?
S14	35	S9 AND (PMAL OR MALTOSE? OR FUSION?)

?t s13/9/all



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## Article



## Current Microbiology

Publisher: Springer-Verlag New York, LLC  
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DOI: 10.1007/s002849900240  
Issue: Volume 35, Number 4  
Date: October 1997  
Pages: 207 - 214

**Molecular Characterization of the Clusters of Genes Encoding the Botulinum Neurotoxin Complex in Clostridium botulinum (Clostridium argentinense) Type G and Nonproteolytic Clostridium botulinum Type B**Manju Bhandari<sup>A1</sup>, Kathryn D. Campbell<sup>A1</sup>, Matthew D. Collins<sup>A1</sup>, Alison K. East<sup>A1</sup>

<sup>A1</sup> Department of Microbiology, Institute of Food Research, Earley Gate, Whiteknights Road, Reading, RG6 6BZ, UK

**Abstract:**

**Abstract.** The cluster of genes encoding components of the progenitor botulinum neurotoxin complex has been mapped and cloned in Clostridium botulinum type G strain ATCC 27322. Determination of the nucleotide sequence of the region has revealed open reading frames encoding nontoxic components of the complex; upstream of the gene encoding BoNT/G (botG). The arrangement of these genes differs from that in strains of other antigenic toxin types. Immediately upstream of botG lies a gene encoding a protein of 1198 amino acids, which shows homology with the nontoxic-nonhemagglutinin (NTNH) component of the progenitor complex. Further upstream there are genes encoding proteins with homology to hemagglutinin components (HA-17, HA-70) and a putative positive regulator of gene expression (P-21). Sequence comparison has shown that BoNT/G has highest homology with BoNT/B. The sequence of the BoNT-cluster of genes in non-proteolytic C. botulinum type B strain Eklund 17B has been extended to include the complete NTNH and HA-17, and partial HA-70 gene sequences. Comparison of NTNH/G with other NTNHs reveals that it shows highest homology with NTNH/B consistent with the genealogical affinity shown between BoNT/G and BoNT/B genes.

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**Structure and function of botulinum toxin]**

Fujii N

Department of Microbiology, Sapporo Medical University School of Medicine, Japan.

Hokkaido igaku zasshi The Hokkaido journal of medical science (JAPAN)

Jan 1995, 70 (1) p19-28, ISSN 0367-6102 Journal Code: 17410290R

Document type: Journal Article; Review; Review, Tutorial ; English Abstract

Languages: JAPANESE

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

**Botulinum** toxins (types A to G) inhibit the release of acetylcholine at the neuromuscular junction. These toxins are produced as progenitor toxins of large molecular sizes of 12S (M toxin), 16S (L toxin) and 19S (LL toxin) in culture supernatants. Three different molecular forms have been demonstrated in **botulinum** type A toxin. L and M toxins are recognized in **botulinum** type C and D toxins. Type E toxin is exclusively composed of M toxin. In an alkaline condition, M and L toxins dissociate into neurotoxin and nontoxic components. Nontoxic components consist of nontoxic-nonhemagglutinin component (nontoxic-nonHA) and hemagglutinin (HA). M toxin is made up by association of neurotoxin with nontoxic-nonHA, and L toxin is formed by conjugation of M toxin with HA. HA also consists of several subcomponents. These **genes** with related functions (progenitor toxin) are closely grouped as operon on the chromosome. Nontoxic-nonHA **gene** is located only 17 bp (type C) or 27 bp (type E) upstream of the neurotoxin **gene**. Both **genes** may be transcribed (right-ward transcription) by a polycistronic mRNA species initiated from a promoter located in the 5'-untranslated region of the nontoxic-nonHA **gene**. The construction of HA subcomponent **genes** (HA-33, HA-17, HA-25 and HA-53) also appears operon structure. The **gene** cluster related HA is located 262 bp upstream of nontoxic-nonHA **gene** of type C and transcribed (left-ward transcription) by the same mRNA from the 5'-noncoding region of HA-33 **gene**. **Botulinum** neurotoxin undergoes cleavage to form a dichain molecule linked through a disulphide bond. The **heavy chain** correlates with the binding of toxin to peripheral synapses, and the **light chain** is associated with the intracellular activity of blocking of acetylcholine release. Fifty amino acids in C- **terminal** region of type C toxin is essential for the binding activity of toxin to the target cells. However, the binding efficiency of type C toxin is not antagonized by the other type of **botulinum** toxins because of low homology of this binding domain of type C toxin to other types. Furthermore, five highly homologous regions are found in **light chain** among seven neurotoxins. One of these homologous regions, sequence HEL-H--, shows strong similarity with the active site of zinc-proteases. The inhibition of acetylcholine release is associated with this protease activity which selectively cleaves the synaptic vesicle membrane proteins. These target membrane proteins are key components of the synaptic vesicle docking and fusion. (ABSTRACT TRUNCATED AT 400 WORDS) (30 Refs.)

Tags: Human

Descriptors: **Botulinum** Toxins--chemistry--CH; Acetylcholine--metabolism --ME; Amino Acid Sequence; Animals; **Botulinum** Toxins-- **genetics** --GE; Molecular Sequence Data; Molecular Weight; Neuromuscular Junction --metabolism--ME

CAS Registry No.: 0 (**Botulinum** Toxins); 51-84-3 (Acetylcholine)

Record Date Created: 19950609

Record Date Completed: 19950609

09445677 PMID: 1388670

**Pepsin fragmentation of botulinum type E neurotoxin: isolation and characterization of 112, 48, 46, and 16 kD fragments.**

Gimenez J A; DasGupta B R

Department of Food Microbiology and Toxicology, University of Wisconsin, Madison 53706.

Journal of protein chemistry (UNITED STATES) Jun 1992, 11 (3) p255-64, ISSN 0277-8033 Journal Code: 8217321

Contract/Grant No.: NS17742; NS; NINDS

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Controlled digestion of approximately 150 kD single chain botulinum type E neurotoxin with pepsin at pH 6.0 produced 112, 48, 46, and 16 kD fragments. These were chromatographically purified; their locations in the approximately 1300 amino acid residue long neurotoxin were determined by identifying the amino terminal 10 residues of 112 and 48 kD fragments, 50 residues of 46 kD fragment, and 59 residues of 16 kD fragment. The 48 and 112 kD fragments contain the N-terminal segment of the neurotoxin (i.e., residue no. 1 to approximately 425 and 1 to approximately 990, respectively), the 46 kD fragment corresponds to approximately 407 residues of the C-terminal region, and the 16 kD fragment contains the approximately 140 residues from a segment nearer to the C-terminus. The 48 kD fragment is similar to the approximately 50 kD N-terminal light chain of the approximately 150 kD dichain neurotoxin, which is generated by tryptic cleavage of the approximately 150 kD single chain neurotoxin, and is separated from the approximately 100 kD C-terminal heavy chain by dithiothreitol (DTT) reduction of an intrachain disulfide bond in the presence of 2 M urea (Sathyamoorthy and DasGupta, J. Biol. Chem. 260, 10461, 1985). The pepsin-generated 48 kD fragment, unlike the light chain, was isolated without exposure to DTT and urea. The single chain 112 kD fragment following trypsin digestion yielded 48 and 60 kD fragments that were separable after DTT reduction of the intrachain disulfide which links them. The N-terminal residues of the smaller fragment were identical to that of the single chain 150 kD neurotoxin; the single chain 112 kD fragment is therefore the neurotoxin minus the approximately 50 kD C-terminal half of the heavy chain. The biological activities of the 48 and 112 kD fragments can be demonstrated in permeabilized PC12 cells (Lomneth et al., J. Neurochem. 57, 1413, 1991); they inhibit norepinephrine release.

Tags: Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

**Molecular cloning of the Clostridium botulinum structural gene encoding the type B neurotoxin and determination of its entire nucleotide sequence.**

Whelan S M; Elmore M J; Bodsworth N J; Brehm J K; Atkinson T; Minton N P  
Division of Biotechnology, PHLS Centre for Applied Microbiology and Research, Salisbury, Wiltshire, United Kingdom.

Applied and environmental microbiology (UNITED STATES) Aug 1992, 58  
(8) p2345-54, ISSN 0099-2240 Journal Code: 7605801

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

DNA fragments derived from the Clostridium botulinum type A neurotoxin (BoNT/A) gene (botA) were used in DNA-DNA hybridization reactions to derive a restriction map of the region of the C. botulinum type B strain Danish chromosome encoding botB. As the one probe encoded part of the BoNT/A heavy (H) chain and the other encoded part of the light (L) chain, the position and orientation of botB relative to this map were established. The temperature at which hybridization occurred indicated that a higher degree of DNA homology occurred between the two genes in the H-chain encoding region. By using the derived restriction map data, a 2.1-kb BglII-XbaI fragment encoding the entire BoNT/B L chain and 108 amino acids of the H chain was cloned and characterized by nucleotide sequencing. A contiguous 1.8-kb XbaI fragment encoding a further 623 amino acids of the H chain was also cloned. The 3' end of the gene was obtained by cloning a 1.6-kb fragment amplified from genomic DNA by inverse polymerase chain reaction. Translation of the nucleotide sequence derived from all three clones demonstrated that BoNT/B was composed of 1,291 amino acids. Comparative alignment of its sequence with all currently characterized BoNTs (A, C, D, and E) and tetanus toxin (TeTx) showed that a wide variation in percent homology occurred dependent on which component of the dichain was compared. Thus, the L chain of BoNT/B exhibits the greatest degree of homology (50% identity) with the TeTx L chain, whereas its H chain is most homologous (48% identity) with the BoNT/A H chain. Overall, the six neurotoxins were shown to be composed of highly conserved amino acid domains interceded with amino acid tracts exhibiting little overall similarity. In total, 68 amino acids of an average of 442 are absolutely conserved between L chains and 110 of 845 amino acids are conserved between H chains. Conservation of Trp residues (one in the L chain and nine in the H chain) was particularly striking. The most divergent region corresponds to the extreme carboxy terminus of each toxin, which may reflect differences in specificity of binding to neurone acceptor sites.

Tags: Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.

**Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A.**

Kurazono H; Mochida S; Binz T; Eisel U; Quanz M; Grebenstein O; Wernars K ; Poulain B; Tauc L; Niemann H

Institute for Microbiology, Federal Research Center for Virus Diseases of Animals, Tübingen, Federal Republic of Germany.

Journal of biological chemistry (UNITED STATES) Jul 25 1992, 267 (21) p14721-9, ISSN 0021-9258 Journal Code: 2985121R

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

To define conserved domains within the **light (L) chains** of clostridial neurotoxins, we determined the sequence of **botulinum neurotoxin type B (BoNT/B)** and aligned it with those of tetanus toxin (TeTx) and BoNT/A, BoNT/C1, BoNT/D, and BoNT/E. The **L chains** of BoNT/B and TeTx share 51.6% identical amino acid residues whereas the degree of identity to other clostridial neurotoxins does not exceed 36.5%. Each of the **L chains** contains a conserved motif, HExxHxxH, characteristic for metalloproteases. We then **generated** specific 5'- and 3'-deletion **mutants** of the **L chain genes** of TeTx and BoNT/A and tested the biological properties of the **gene** products by microinjection of the corresponding mRNAs into identified presynaptic cholinergic neurons of the buccal ganglia of *Aplysia californica*. Toxicity was determined by measurement of neurotransmitter release, as detected by depression of postsynaptic responses to presynaptic stimuli (Mochida, S., Poulain, B., Eisel, U., Binz, T., Kurazono, H., Niemann, H., and Tauc, L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7844-7848). Our studies allow the following conclusions. 1) Residues Cys439 of TeTx and Cys430 of BoNT/A, both of which participate in the interchain disulfide bond, play no role in the toxification reaction. 2) ~~Derivatives of TeTx that lacked either 8-amino- or 65-carboxyl-~~ **terminal** residues are still toxic, whereas those lacking 10 amino- or 68 carboxyl- **terminal** residues are nontoxic. 3) For BoNT/A, toxicity could be demonstrated only in the presence of added nontoxic **heavy (H) chain**. A deletion of 8 amino- **terminal** or 32 carboxyl- **terminal** residues from the **L chain** had no effect on toxicity, whereas a removal of 10 amino- **terminal** or 57 carboxyl- **terminal** amino acids abolished toxicity. 4) The synergistic effect mediated by the **H chain** is linked to the carboxyl- **terminal** portion of the **H chain**, as demonstrated by injection of HC-specific mRNA into neurons containing the **L chain**. This finding suggests that the HC domain of the **H chain** becomes exposed to the cytosol during or after the putative translocation step of the **L chain**.

Tags: Support, Non-U.S. Gov't

Descriptors: **Botulinum** Toxins--toxicity--TO; \*Tetanus Toxin--toxicity--TO; Acetylcholine--metabolism--ME; Amino Acid Sequence; Animals; *Aplysia*; Base Sequence; **Botulinum** Toxins--**genetics**--GE; Depression, Chemical; **Genetic** Vectors; Microinjections; Molecular Sequence Data; Mutation; RNA, Messenger--metabolism--ME; Sequence Alignment; Tetanus Toxin--**genetics**--GE; Transcription, **Genetic**; Translation, **Genetic**; *Xenopus*

CAS Registry No.: 0 (Botulinum Toxins); 0 (Genetic Vectors); 0 (RNA, Messenger); 0 (Tetanus Toxin); 51-84-3 (Acetylcholine)

Record Date Created: 19920826

Record Date Completed: 19920826

**Exogenous mRNA encoding tetanus or botulinum neurotoxins expressed in Aplysia neurons.**

Mochida S; Poulain B; Eisel U; Binz T; Kurazono H; Niemann H; Tauc L  
Laboratoire de Neurobiologie Cellulaire et Moléculaire, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France.

Proceedings of the National Academy of Sciences of the United States of America (UNITED STATES) Oct 1990, 87 (20) p7844-8, ISSN 0027-8424

Journal Code: 7505876

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Injection of exogenous mRNA purified from various tissue preparations into cellular translation systems such as *Xenopus* oocytes has allowed expression of complex proteins (e.g., receptors for neurotransmitters). No evidence for expression of injected exogenous mRNA, however, has been reported in **terminally** differentiated neurons. If achieved, it would allow the study of long-lasting changes of properties of nerve cells in their functional context. To obtain evidence of such expression, we chose two proteins that produce a detectable effect even at very low intracellular concentrations. Tetanus toxin and **botulinum** neurotoxin fulfill this criterion, being the most potent neurotoxins known. Both toxins block neurotransmitter release at nanomolar intracellular concentrations. These di-**chain** proteins, consisting of a **light chain** and a **heavy chain**, have recently been sequenced. Their active sites are located (or partly located) on the **light chain**. mRNAs encoding the **light chain** of either toxin were transcribed in vitro from the cloned and specifically truncated **genes** of *Clostridium tetani* and *Clostridium botulinum*, respectively, and injected into presynaptic cholinergic neurons of the buccal ganglia of *Aplysia californica*. Depression of neurotransmitter release appeared in less than 1 hr, demonstrating successful expression of foreign mRNA injected into a neuron in situ.

Tags: Female; Support, Non-U.S. Gov't

Descriptors: **Botulinum** Toxins-- **genetics** --GE; \*Neurons--physiology--PH ; \*Neurotoxins; \*RNA, Messenger-- **genetics** --GE; \*Tetanus Toxin-- **genetics** --GE; Animals; Aplysia; Macromolecular Systems; Microinjections; Oocytes --physiology--PH; RNA, Messenger--administration and dosage--AD; Translation, **Genetic** ; *Xenopus laevis*

CAS Registry No.: 0 (Botulinum Toxins); 0 (Macromolecular Systems); 0 (Neurotoxins); 0 (RNA, Messenger); 0 (Tetanus Toxin)

Record Date Created: 19901204

Record Date Completed: 19901204

09032326 PMID: 1910014

**Characterization of the C3 gene of Clostridium botulinum types C and D and its expression in Escherichia coli.**

Popoff M R; Hauser D; Boquet P; Eklund M W; Gill D M

Unite des Antigenes Bacteriens, Institut Pasteur, Paris, France.

Infection and immunity (UNITED STATES) Oct 1991, 59 (10) p3673-9,

ISSN 0019-9567 Journal Code: 0246127

Contract/Grant No.: AI16928; AI; NIAID

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Clostridium botulinum type C and D strains produce exoenzyme C3, which ADP-ribosylates the Rho protein, a 21-kDa regulatory GTP-binding protein. In a previous work, we demonstrated that the C3 gene is encoded by bacteriophages C and D of C. botulinum by using DNA-DNA hybridizations with oligonucleotides deduced from the C3 protein N-terminal sequence. The C3 coding gene was cloned and sequenced, but its upstream DNA region could not be studied because of its instability in Escherichia coli. In this work, the upstream DNA region of the C3 gene was directly amplified by the polymerase chain reaction and sequenced. The C3 gene encodes a polypeptide of 251 amino acids (27,823 Da) consisting of a 40-amino-acid signal peptide and a mature protein of 211 amino acids (23,546 Da). The C3 mature protein was expressed in E. coli under the control of the trc promoter. The recombinant polypeptide obtained was recognized by C3 antibodies and ADP-ribosylated the Rho protein. The C3 gene nucleotide sequence is identical on C and D phage DNAs. At the amino acid sequence level, no similarity was found among C3, other ADP-ribosylating toxins, or tetanus or botulin A, C1, and D neurotoxins.

Tags: Support, U.S. Gov't, Non-P.H.S.; Support, U.S. Gov't, P.H.S.

Descriptors: Bacteriophages-- genetics --GE; \*Clostridium botulinum -- genetics --GE; \*Escherichia coli-- genetics --GE; \*Gene Expression; \*Genes , Bacterial; \*Poly(ADP-ribose) Polymerases-- genetics --GE; Amino Acid Sequence; Base Sequence; Molecular Sequence Data; Protein Sorting Signals--analysis--AN; Recombinant Fusion Proteins--analysis--AN

Molecular Sequence Databank No.: GENBANK/S61978; GENBANK/S61981; GENBANK/S70201; GENBANK/S70204; GENBANK/X06392; GENBANK/X14442; GENBANK/X16435; GENBANK/X53033; GENBANK/X57105; GENBANK/X59039; GENBANK/X59040; GENBANK/X59240

CAS Registry No.: 0 (Protein Sorting Signals); 0 (Recombinant Fusion Proteins)

Enzyme No.: EC 2.4.2.30 (Poly(ADP-ribose) Polymerases)

Record Date Created: 19911021

Record Date Completed: 19911021

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
24 October 2002 (24.10.2002)

PCT

(10) International Publication Number  
**WO 02/083179 A2**

- (51) International Patent Classification<sup>7</sup>: **A61K 47/48**
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2,362,004 13 November 2001 (13.11.2001) CA  
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- (71) Applicant: **BIOAXONE THERAPEUTIQUE INC.**  
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- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
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- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: FUSION PROTEINS

(57) Abstract: The Rho family GTPases regulates axon growth and regeneration. Inactivation of Rho with C3, a toxin from *Clostridium botulinum*, can stimulate regeneration and sprouting of injured axons. The present invention provides novel chimeric C3-like Rho antagonists. These new antagonists are a significant improvement over C3 compounds because they are 3-4 orders of magnitude more potent to stimulate axon growth on inhibitory substrates than recombinant C3. The invention further provides evidence that these compounds promote repair when applied to the injured mammalian central nervous system.

**TITLE: FUSION PROTEINS****FIELD OF THE INVENTION**

5 The present invention relates to conjugate or fusion type proteins (polypeptides) comprising, for example, C3 (see below) (i.e., C3-like protein, C3 chimeric proteins). Although, in the following, fusion-type proteins of the present invention, will be particularly discussed in relation to the use to facilitate regeneration of axons and neuroprotection, it is to be understood that the fusion proteins may be exploited in other contexts.

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The present invention in particular pertains to the field of mammalian nervous system repair (e.g. repair of a central nervous system (CNS) lesion site or a peripheral nervous system (PNS) lesion site), axon regeneration and axon sprouting, neurite growth and protection from neurodegeneration and ischemic damage.

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The Rho family GTPases regulates axon growth and regeneration. Inactivation of Rho with *Clostridium botulinum* C3 exotransferase (hereinafter simply referred to as C3) can stimulate regeneration and sprouting of injured axons; C3 is a toxin purified from *Clostridium botulinum* (see Saito et al., 1995, FEBS Lett 371:105-109; Wilde et al 2000. J. Biol. Chem. 275:16478). Compounds of the C3 family from *Clostridium botulinum* inactivate Rho by ADP-ribosylation and thus act as antagonists of Rho effect or function (Rho antagonists).

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The present invention in particular relates to a means of intracellular delivery of C3 protein (e.g. C3 itself or other active analogues such as C3-like transferases - see below) or other Rho antagonists to repair damage in the nervous system, to prevent ischemic cell death, and to treat various disease where the inactivation of Rho is required. The means of delivery may take the form of chimeric (i.e. conjugate) C3-like Rho antagonists. These conjugate antagonists provide a significant improvement over C3 compounds (alone) because they are 3 to 4 orders of magnitude more potent with respect to the stimulation of axon growth on inhibitory substrates than recombinant C3 alone. Examples of these Rho antagonists have been made as recombinant proteins created to facilitate penetration of the cell membrane (i.e.

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I claim:

1. A drug delivery construct comprising at least one transport agent region and an active agent region, wherein the transport agent region is able to facilitate the uptake  
5 of the active agent region into a cell, and wherein the active agent region is an active therapeutic agent region able to facilitate axon growth, and an analogue thereof.
2. A drug delivery construct as defined in claim 1 wherein the active agent region is selected from the group consisting of ADP-ribosyl transferase C3 and ADP-ribosyl  
10 transferase C3 analogue thereof.
3. A drug conjugate consisting of a transport polypeptide moiety covalently linked to an active cargo moiety wherein the transport polypeptide moiety is able to facilitate the uptake of the active cargo moiety into a mammalian tissue or cell and wherein the  
15 active cargo moiety is an active therapeutic moiety able to facilitate axon growth.
4. A drug conjugate as defined in claim 3, wherein the transport polypeptide moiety  
is selected from the group consisting of a transport subdomain of HIV Tat protein, a homeodomain of antennapedia, a Histidine tag and analogues thereof and wherein the  
20 active cargo moiety is selected from the group consisting of C3 protein able to facilitate axon growth.
5. A drug conjugate as defined in claim 4 wherein the C3 protein is selected from the group consisting of ADP-ribosyl transferase C3 and ADP-ribosyl transferase C3  
25 analogues thereof.
6. A drug conjugate as defined in claim 3 wherein the transport polypeptide moiety includes an active contiguous amino acid sequence as described herein.
- 30 7. The use of a drug delivery construct as defined in claim 1, and analogues thereof for suppressing the inhibition of neuronal axon growth.
8. The use of a drug delivery construct as defined in claim 2, and analogues thereof

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A01N 63/00, C12N 15/64, 5/00, A61K 38/00, A01N 43/04</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/08533</b> <b>(43) International Publication Date:</b> 25 February 1999 (25.02.99)
<b>(21) International Application Number:</b> PCT/US98/16794 <b>(22) International Filing Date:</b> 12 August 1998 (12.08.98)  <b>(30) Priority Data:</b> 60/055,268 13 August 1997 (13.08.97) US  <b>(71) Applicant (for all designated States except US):</b> YALE UNIVERSITY [US/US]; Office of Cooperative Research, 155 Whitney Avenue, New Haven, CT 06520-8336 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> STRITTMATTER, Stephen, M. [US/US]; 26 Pleasant Valley Road, Clinton, CT 06413 (US).  <b>(74) Agent:</b> KRINSKY, Mary, M.; 88 Prospect Street, New Haven, CT 06511-3797 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> CENTRAL NERVOUS SYSTEM AXON REGENERATION  <b>(57) Abstract</b>  Therapies for the treatment of a variety of central nervous system injuries including acute or chronic spinal cord injury, traumatic brain injury, and white matter stroke involve the administration of rho protein inhibitors to promote axon regeneration. Local administration is employed in typical embodiments, and this may include injection of a recombinant virus that expresses an inhibitor. In one embodiment, the inhibitor is <i>C. botulinum</i> C3 exoenzyme or a chimeric <i>C. botulinum</i> C2/C3 construct expressed in a replication-deficient adeno, adeno-associated, or herpes virus.		



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 39/00, 39/38, 38/08, C12P 21/06, 21/04, 21/08, C12N 15/00, 15/09, 15/63, 15/70, 15/74, C07K 16/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/08540</b> <b>(43) International Publication Date:</b> 5 March 1998 (05.03.98)
<b>(21) International Application Number:</b> PCT/US97/15394 <b>(22) International Filing Date:</b> 28 August 1997 (28.08.97)  <b>(30) Priority Data:</b> 08/704,159 28 August 1996 (28.08.96) US  <b>(71) Applicant:</b> OPHIDIAN PHARMACEUTICALS, INC. [US/US]; 5445 East Cheryl Parkway, Madison, WI 53711 (US).  <b>(72) Inventors:</b> WILLIAMS, James, A.; 6420 Pueblo Court, Lincoln, NE 68516 (US). THALLEY, Bruce, S.; 126 Marinette Trail, Madison, WI 53705 (US).  <b>(74) Agents:</b> CARROLL, Peter, G. et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> MULTIVALENT VACCINE FOR <i>CLOSTRIDIUM BOTULINUM</i> NEUROTOXIN  <b>(57) Abstract</b>  <p>The present invention includes recombinant proteins derived from <i>Clostridium botulinum</i> toxins. In particular, soluble recombinant <i>Clostridium botulinum</i> type A, type B and type E toxin proteins are provided. Methods which allow for the isolation of recombinant proteins free of significant endotoxin contamination are provided. The soluble, endotoxin-free recombinant proteins are used as immunogens for the production of vaccines and antitoxins. These vaccines and antitoxins are useful in the treatment of humans and other animals at risk of intoxication with clostridial toxin.</p>		

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/15394

A. CLASSIFICATION OF SUBJECT MATTER:  
IPC (6):

A61K 39/00, 39/38, 38/08; C12P 21/06, 21/04, 21/08; C12N 15/00, 15/09, 15/63, 15/70, 15/74; C07K 16/00

A. CLASSIFICATION OF SUBJECT MATTER:  
US CL :

424/184.1, 192.1, 247.1; 435/69.1, . 69.7, 325, 320.1; 530/388.4, 389.5



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification<sup>6</sup> : <b>C12N 15/31, 1/21, C12P 21/02, C07K 14/33, A61K 38/16, 39/08</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 98/07864</b></p> <p>(43) International Publication Date: 26 February 1998 (26.02.98)</p>
<p>(21) International Application Number: <b>PCT/GB97/02273</b></p> <p>(22) International Filing Date: 22 August 1997 (22.08.97)</p> <p>(30) Priority Data:              9617671.4                      23 August 1996 (23.08.96)      GB              9625996.5                      13 December 1996 (13.12.96)      GB</p> <p>(60) Parent Application or Grant          (63) Related by Continuation              US    08/782,893 (CIP)              Filed on                                      27 December 1996 (27.12.96)</p> <p>(71) Applicants (for all designated States except US): MICROBIOLOGICAL RESEARCH AUTHORITY CAMR (CENTRE FOR APPLIED MICROBIOLOGY &amp; RESEARCH) [GB/GB]; Porton Down, Salisbury, Wiltshire SP4 0JG (GB). THE SPEYWOOD LABORATORY LIMITED [GB/GB]; 14 Kensington Square, London W8 5HH (GB).</p> <p>(72) Inventors; and          (75) Inventors/Applicants (for US only): SHONE, Clifford, Charles [GB/GB]; Microbiological Research Authority CAMR (Centre for Applied Microbiology &amp; Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). QUINN,</p>	<p>Conrad, Padraig [GB/GB]; Microbiological Research Authority CAMR (Centre for Applied Microbiology &amp; Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). FOSTER, Keith, Alan [GB/GB]; Microbiological Research Authority CAMR (Centre for Applied Microbiology &amp; Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB).</p> <p>(74) Agent: SCHLICH, George, William; Mathys &amp; Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b>  <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: RECOMBINANT TOXIN FRAGMENTS</p> <p>(57) Abstract</p> <p>A polypeptide has first and second domains which enable the polypeptide to be translocated into a target cell or which increase the solubility of the polypeptide, or both, and further enable the polypeptide to cleave one or more vesicle or plasma-membrane associated proteins essential to exocytosis. The polypeptide thus combines useful properties of a clostridial toxin, such as a botulinum or tetanus toxin, without the toxicity associated with the natural molecule. The polypeptide can also contain a third domain that targets it to a specific cell, rendering the polypeptide useful in inhibition of exocytosis in target cells. Fusion proteins comprising the polypeptide, nucleic acids encoding the polypeptide and methods of making the polypeptide are also provided. Controlled activation of the polypeptide is possible and the polypeptide can be incorporated into vaccines and toxin assays.</p>		

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 97/02273

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C12N1/21 C12P21/02 C07K14/33 A61K38/16  
A61K39/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 12802 A (OPHIDIAN PHARM INC ; WILLIAMS JAMES A (US); PADHYE NISHA V (US); KI) 2 May 1996 see the whole document ---	1-52
X	KURAZONO H ET AL: "Minimal essential *domains* specifying toxicity of the *light* *chains* of tetanus toxin and botulinum neurotoxin type A." J BIOL CHEM, JUL 25 1992, 267 (21) P14721-9, UNITED STATES, XP002047910 see table II --- -/-	1-52

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

\*A\* document defining the general state of the art which is not considered to be of particular relevance

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\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

9 December 1997

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: <b>PCT/US00/12890</b>		(74) Agents: POSORSKE, Laurence, H. et al.; Baker Botts, L.L.P., The Warner, 1299 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).	
(22) International Filing Date: 12 May 2000 (12.05.00)			
(30) Priority Data: 60/133,866 12 May 1999 (12.05.99) US 60/133,873 12 May 1999 (12.05.99) US 60/133,869 12 May 1999 (12.05.99) US 60/133,865 12 May 1999 (12.05.99) US 60/133,868 12 May 1999 (12.05.99) US 60/133,867 12 May 1999 (12.05.99) US 60/146,192 29 July 1999 (29.07.99) US		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
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(72) Inventors; and (75) Inventors/Applicants (for US only): SMITH, Leonard, A. [US/US]; Clarksburg, MD (US). BYRNE, Michael, P. [US/US]; Frederick, MD (US). MIDDLEBROOK, John, L. [US/US]; Middletown, MD (US). LAPENOTIERE, Hugh [US/US]; Charlestown, WV (US).			
(54) Title: RECOMBINANT VACCINE AGAINST BOTULINUM NEUROTOXIN			
(57) Abstract <p>This invention is directed to preparation and expression of synthetic genes encoding polypeptides containing protective epitopes of botulinum neurotoxin (BoNT). The invention is also directed to production of immunogenic peptides encoded by the synthetic genes, as well as recovery and purification of the immunogenic peptides from recombinant organisms. The invention is also directed to methods of vaccination against botulism using the expressed peptides.</p>			



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 39/00, 39/38, 38/08, C12P 21/06, 21/04, 21/08, C12N 15/00, 15/09, 15/63, 15/70, 15/74, C07K 16/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/08540</b> <b>(43) International Publication Date:</b> 5 March 1998 (05.03.98)
<b>(21) International Application Number:</b> PCT/US97/15394 <b>(22) International Filing Date:</b> 28 August 1997 (28.08.97)  <b>(30) Priority Data:</b> 08/704,159                      28 August 1996 (28.08.96)                      US  <b>(71) Applicant:</b> OPHIDIAN PHARMACEUTICALS, INC. [US/US]; 5445 East Cheryl Parkway, Madison, WI 53711 (US).  <b>(72) Inventors:</b> WILLIAMS, James, A.; 6420 Pueblo Court, Lincoln, NE 68516 (US). THALLEY, Bruce, S.; 126 Marinette Trail, Madison, WI 53705 (US).  <b>(74) Agents:</b> CARROLL, Peter, G. et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> <del>MULTIVALENT VACCINE FOR CLOSTRIDIUM BOTULINUM NEUROTOXIN</del>  <b>(57) Abstract</b> <p>The present invention includes recombinant proteins derived from <i>Clostridium botulinum</i> toxins. In particular, soluble recombinant <i>Clostridium botulinum</i> type A, type B and type E toxin proteins are provided. Methods which allow for the isolation of recombinant proteins free of significant endotoxin contamination are provided. The soluble, endotoxin-free recombinant proteins are used as immunogens for the production of vaccines and antitoxins. These vaccines and antitoxins are useful in the treatment of humans and other animals at risk of intoxication with clostridial toxin.</p>		



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## United States Patent [19]

Kink et al.

[11] Patent Number: 5,736,139

[45] Date of Patent: Apr. 7, 1998

[54] TREATMENT OF *CLOSTRIDIUM DIFFICILE* INDUCED DISEASE

[75] Inventors: John A. Kink; Bruce S. Thalley; Douglas C. Stafford, all of Madison, Wis.; Joseph R. Firca, Vernon Hills, Ill.; Nisha V. Padhye, Madison, Wis.

[73] Assignee: Ochidian Pharmaceuticals, Inc., Madison, Wis.

[21] Appl. No.: 480,604

[22] Filed: Jun. 7, 1995

## Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 422,711, Apr. 14, 1995, which is a continuation-in-part of Ser. No. 405,496, Mar. 16, 1995, which is a continuation-in-part of Ser. No. 329,154, Oct. 24, 1994, which is a continuation-in-part of Ser. No. 161,907, Dec. 2, 1993, Pat. No. 5,601,823, which is a continuation-in-part of Ser. No. 985,321, Dec. 4, 1992, which is a continuation-in-part of Ser. No. 429,791, Oct. 31, 1989, Pat. No. 5,196,193.

[51] Int. Cl.<sup>6</sup> ..... A61K 39/395; C07K 16/12

[52] U.S. Cl. .... 424/164.1; 424/167.1; 530/389.1; 530/389.5

[58] Field of Search ..... 424/164.1, 167.1; 530/389.1, 389.5

## [56] References Cited

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Attorney, Agent, or Firm—Medlen & Carroll, LLP

## [57] ABSTRACT

The present provides neutralizing antitoxin directed against *C. difficile* toxins. These antitoxins are produced in arian species using soluble recombinant *C. difficile* toxin proteins. The avian antitoxins are designed so as to be orally administrable in therapeutic amounts and may be in any form (i.e., as a solid or in aqueous solution). Solid forms of the antitoxin may comprise an enteric coating. These antitoxins are useful in the treatment of humans and other animals intoxicated with at least one bacterial toxin. The invention further provides vaccines capable of protecting a vaccinated recipient from the morbidity and mortality associated with *C. difficile* infection. These vaccines are useful for administration to humans and other animals at risk of exposure to *C. difficile* toxins.

28 Claims, 53 Drawing Sheets

First Hit

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Jul 22, 2004

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TITLE: Recombinant botulinum toxins having a soluble C-terminal portion of a heavy chain, an N-terminal portion of a heavy chain and a light chain

PUBLICATION-DATE: July 22, 2004

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APPL-NO: 10/ 729039 [PALM]

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REPRESENTATIVE-FIGURES: NONE

## ABSTRACT:

The present invention includes recombinant proteins derived from Clostridium botulinum toxins. In particular, soluble recombinant Clostridium botulinum type A, type B and type E toxin proteins are provided. Methods which allow for the isolation of recombinant proteins free of significant endotoxin contamination are provided. The soluble, endotoxin-free recombinant proteins are used as immunogens for the production of vaccines and antitoxins. These vaccines and antitoxins are useful in the treatment of humans and other animals at risk of intoxication with clostridial toxin.

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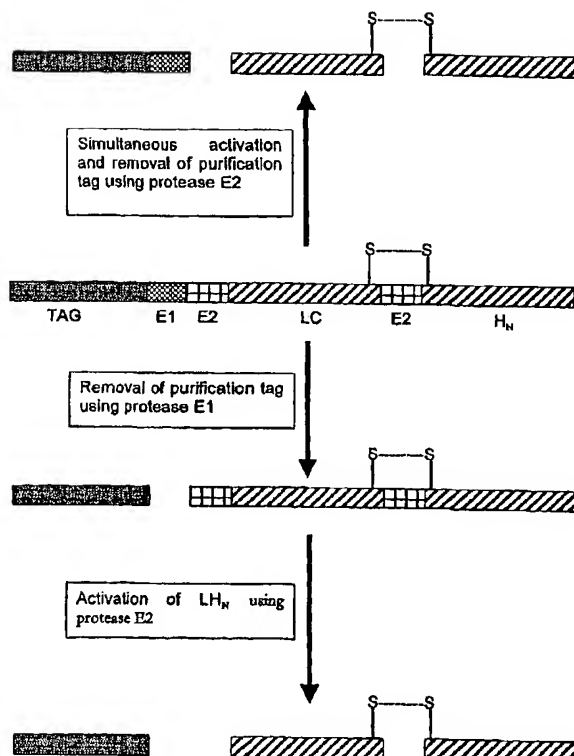
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[Continued on next page]

(54) Title: RECOMBINANT TOXIN FRAGMENTS



(57) Abstract: A single polypeptide is provided which comprises first and second domains. The first domain enables the polypeptide to cleave one or more vesicle or plasma-membrane associated proteins essential to exocytosis, and the second domain enables the polypeptide to be translocated into a target cell or increases the solubility of the polypeptide, or both. The polypeptide thus combines useful properties of a clostridial toxin, such as a botulinum or tetanus toxin, without the toxicity associated with the natural molecule. The polypeptide can also contain a third domain that targets it to a specific cell, rendering the polypeptide useful in inhibition of exocytosis in target cells. Fusion proteins comprising the polypeptide, nucleic acids encoding the polypeptide and methods of making the polypeptide are also provided. Controlled activation of the polypeptide, is possible and the polypeptide can be incorporated into vaccines and toxin assays.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

## RECOMBINANT TOXIN FRAGMENTS

This invention relates to recombinant toxin fragments, to DNA encoding these fragments and to their uses such as in a vaccine and for *in vitro* and *in vivo* purposes.

The clostridial neurotoxins are potent inhibitors of calcium-dependent neurotransmitter secretion in neuronal cells. They are currently considered to mediate this activity through a specific endoproteolytic cleavage of at least one of three vesicle or pre-synaptic membrane associated proteins VAMP, syntaxin or SNAP-25 which are central to the vesicle docking and membrane fusion events of neurotransmitter secretion. The neuronal cell targeting of tetanus and botulinum neurotoxins is considered to be a receptor mediated event following which the toxins become internalised and subsequently traffic to the appropriate intracellular compartment where they effect their endopeptidase activity.

The clostridial neurotoxins share a common architecture of a catalytic L-chain (LC, ca 50 kDa) disulphide linked to a receptor binding and translocating H-chain (HC, ca 100 kDa). The HC polypeptide is considered to comprise all or part of two distinct functional domains. The carboxy-terminal half of the HC (ca 50 kDa), termed the H<sub>C</sub> domain, is involved in the high affinity, neurospecific binding of the neurotoxin to cell surface receptors on the target neuron, whilst the amino-terminal half, termed the H<sub>N</sub> domain (ca 50 kDa), is considered to mediate the translocation of at least some portion of the neurotoxin across cellular membranes such that the functional activity of the LC is expressed within the target cell. The H<sub>N</sub> domain also has the property, under conditions of low pH, of forming ion-permeable channels in lipid membranes, this may in some manner relate to its translocation function.

For botulinum neurotoxin type A (BoNT/A) these domains are considered to reside within amino acid residues 872-1296 for the H<sub>C</sub>, amino acid residues 449-871 for the H<sub>N</sub> and residues 1-448 for the LC. Digestion with trypsin effectively degrades the H<sub>C</sub> domain of the BoNT/A to generate a non-toxic fragment designated LH<sub>N</sub>, which is no longer able to bind to and enter neurons (Fig. 1). The LH<sub>N</sub> fragment so produced also has the property

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**CLAIMS**

1. A single chain polypeptide comprising first and second domains, wherein:-  
said first domain is a clostridial neurotoxin light chain or a fragment or a variant thereof, wherein said first domain is capable of cleaving one or more vesicle or plasma membrane associated proteins essential to exocytosis; and  
said second domain is a clostridial neurotoxin heavy chain H<sub>N</sub> portion or a fragment or a variant thereof, wherein said second domain is capable of (i) translocating the polypeptide into a cell or (ii) increasing the solubility of the polypeptide compared to the solubility of the first domain on its own or (iii) both translocating the polypeptide into a cell and increasing the solubility of the polypeptide compared to the solubility of the first domain on its own; and said second domain lacks a functional C-terminal part of a clostridial neurotoxin heavy chain designated H<sub>C</sub> thereby rendering the polypeptide incapable of binding to cell surface receptors that are the natural cell surface receptors to which native clostridial neurotoxin binds; and wherein said single chain polypeptide comprises a sequence selected from the group consisting of:-  
(I) SEQ ID NO: 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 139, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, and 175; or  
(II) a fragment or variant of (I) having a first domain that is capable of cleaving one or more vesicle or plasma membrane associated proteins essential to exocytosis.
2. A polypeptide according to Claim 1 wherein said clostridial toxin heavy chain is a botulinum neurotoxin heavy chain.
3. A polypeptide according to Claim 1 wherein said clostridial toxin heavy chain is a tetanus neurotoxin heavy chain.

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4. A polypeptide according to any preceding claim, wherein the first domain exhibits endopeptidase activity specific for a substrate selected from one or more of SNAP-25, synaptobrevin/VAMP and syntaxin.
  5. A polypeptide according to any preceding claim, wherein said second domain is a clostridial toxin heavy chain H<sub>N</sub> portion.
  6. A polypeptide according to Claim 1, wherein said clostridial neurotoxin heavy chain is a botulinum neurotoxin type A chain.
  7. A polypeptide according to Claim 1, wherein the second domain comprises the 423 N-terminal amino acids of botulinum toxin type A heavy chain.
  8. A polypeptide according to Claim 1, wherein said clostridial neurotoxin heavy chain is a botulinum neurotoxin type B chain.
- 
9. A polypeptide according to Claim 1, wherein the second domain comprises the 107 N-terminal amino acids of a botulinum toxin type B heavy chain.
  10. A polypeptide according to Claim 1, wherein the second domain comprises the 417 N-terminal amino acids of botulinum toxin type B heavy chain.
  11. A polypeptide according to Claim 1 wherein the second domain comprises the 422 N-terminal amino acids of tetanus heavy chain.
  12. A polypeptide according to Claim 1 wherein the second domain comprises the 100 N-terminal amino acids of a clostridial neurotoxin heavy chain.
  13. A polypeptide according to Claim 1 comprising a site for cleavage by a proteolytic enzyme.
  14. A polypeptide according to Claim 13, wherein the cleavage site is not present in

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a native clostridial neurotoxin.

15. A polypeptide according to Claim 13 or Claim 14, wherein the cleavage site allows proteolytic cleavage of the first and second domains.

16. A polypeptide according to any of Claims 13-15, wherein the cleavage site allows proteolytic cleavage of the first and second domains, and when so cleaved said first domain exhibits greater enzyme activity in cleaving said one or more vesicle or plasma membrane associated protein than does the polypeptide prior to said proteolytic cleavage.

17. A polypeptide according to any of Claims 13-16 obtainable by providing a first nucleic acid sequence encoding said cleavage site within a second nucleic acid sequence encoding a peptide according to Claim 1.

18. A polypeptide according to any preceding claim, wherein the second domain lacks a C-terminal part of a clostridial neurotoxin heavy chain designated Hc.

19. A polypeptide according to any preceding claim, further comprising a third domain that binds the polypeptide to a cell, by binding of the third domain directly to a cell or by binding of the third domain to a ligand or to ligands that bind to a cell.

20. A polypeptide according to Claim 19, wherein said third domain is for binding the polypeptide to an immunoglobulin.

21. A polypeptide according to Claim 20, wherein said third domain is a tandem repeat synthetic IgG binding domain derived from domain b of Staphylococcal protein A.

22. A polypeptide according to Claim 19, wherein said third domain comprises an amino acid sequence that binds to a cell surface receptor.

23. A polypeptide according to Claim 22, wherein said third domain is insulin-like

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growth factor-1 (IGF-1).

24. A polypeptide according to any preceding claim including a spacer molecule between the first and second domains.

25. A polypeptide according to any of Claims 19-23 including a spacer molecule between the second and third domains.

26. A polypeptide according to any preceding claim, further comprising a purification tag that binds to an affinity matrix thereby facilitating purification of the polypeptide using said matrix.

27. A polypeptide according to Claim 26 including a spacer molecule between the purification tag and the polypeptide.

28. A polypeptide according to Claim 26 or Claim 27, wherein said purification tag binds to an affinity matrix of glutathione sepharose.

29. A polypeptide according to any of Claims 26-28, wherein a first protease cleavage site is incorporated between the polypeptide according to any of Claim 1-23 and the purification tag, said protease cleavage site enabling proteolytic separation of said polypeptide from said purification tag.

30. A polypeptide according to any of Claims 26-29, wherein a second proteolytic cleavage site is incorporated between the first and second domains of the polypeptide according to any of Claims 1-23, said protease cleavage site enabling proteolytic cleavage of the first and second domains.

31. A nucleic acid encoding a polypeptide according to any preceding claim.

32. A nucleic acid according to Claim 31, wherein said nucleic acid lacks nucleotides encoding a portion designated H<sub>c</sub> of a clostridial neurotoxin.

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33. A nucleic acid according to Claim 31 or Claim 32, comprising nucleotides encoding residues 1-423 of a botulinum toxin type A heavy chain H<sub>N</sub> domain.

34. A nucleic acid according to Claim 31 or Claim 32, comprising nucleotides encoding residues 1-417 of a botulinum toxin type B heavy chain H<sub>N</sub> domain.

35. A nucleic acid according to any of Claims 31-34, comprising nucleotides encoding a proteolytic cleavage site.

36. A nucleic acid according to Claim 35, wherein the proteolytic cleavage site is not present in a native clostridial neurotoxin.

37. A nucleic acid according to Claim 36, wherein said proteolytic cleavage site is located between the first and second domains of the polypeptide.

---

38. A nucleic acid according to Claim 36 or 37, obtainable by providing a nucleic acid sequence encoding said cleavage site within a nucleic acid sequence according to Claim 29.

39. A nucleic acid sequence selected from the group consisting of:- SEQ ID 69, 71, 73, 75, 77, 113, 134, or a fragment or variant thereof.

40. A single chain polypeptide selected from the group consisting of:- SEQ ID 70, 72, 74, 76, 78, 114, or a fragment or variant thereof.

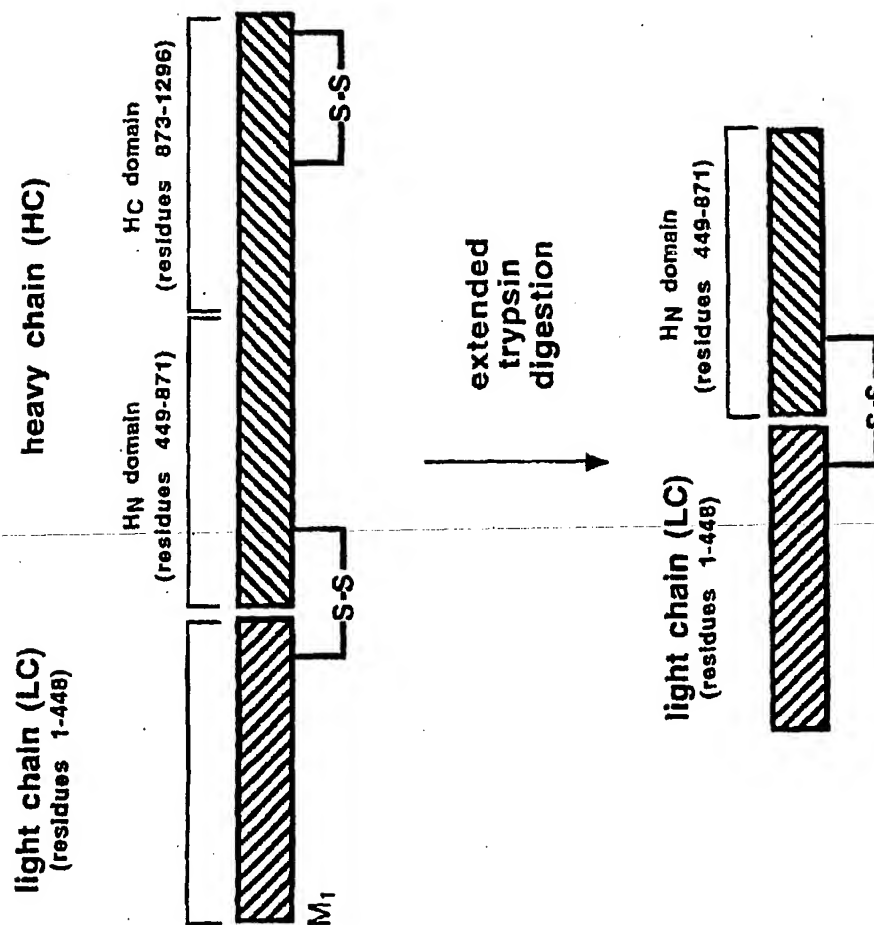


FIG. 1

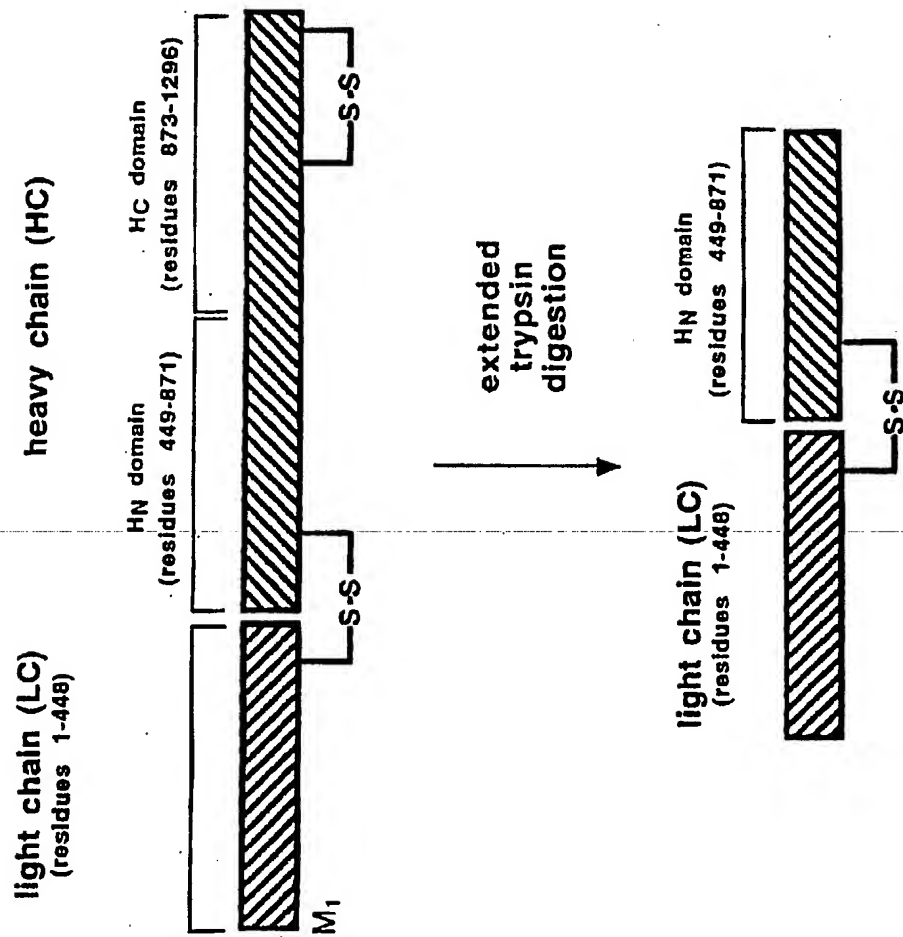
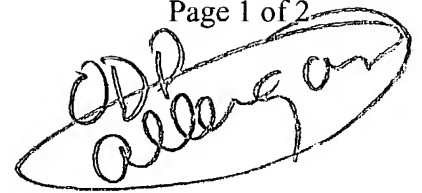


FIG.1

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L5: Entry 1 of 19

File: USPT

Sep 7, 2004

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DOCUMENT-IDENTIFIER: US 6787517 B1

TITLE: Agent and methods for treating pain

DATE-ISSUED: September 7, 2004

## INVENTOR-INFORMATION:

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Aoki; Kei R.	Coto de Caza	CA		

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APPL-NO: 09/ 751053   [PALM]

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FIELD-OF-SEARCH: 514/1, 514/2, 514/14

PRIOR-ART-DISCLOSED:

## U.S. PATENT DOCUMENTS

PAT-NO	ISSUE-DATE	PATENTEE-NAME	US-CL
<input type="checkbox"/> <u>5223408</u>	June 1993	Goeddel et al.	
<input type="checkbox"/> <u>5595880</u>	January 1997	Weinshank et al.	435/7.21
<input type="checkbox"/> <u>5989545</u>	November 1999	Foster et al.	
<input type="checkbox"/> <u>6641820</u>	November 2003	Donovan	

## FOREIGN PATENT DOCUMENTS

FOREIGN-PAT-NO	PUBN-DATE	COUNTRY	US-CL
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WO 96/01813	January 1996	WO
WO96/33273	October 1996	WO
WO01/78702	October 2001	WO

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Kolasa et al., "Alpha-1 Adrenergic Antagonist Effect on Cholinergic Muscarinic Receptors", Society for Neuroscience Abstract, vol. 27, No. 1, 2001, p. 1458.

ART-UNIT: 1637

PRIMARY-EXAMINER: Riley; Jezia

ATTY-AGENT-FIRM: Stout, Uxa, Buyan & Mullins, LLP Uxa; Frank J. Hollrigel; Greg S.

## ABSTRACT:

Agents for treating pain, methods for producing the agents and methods for treating pain by administration to a patient of a therapeutically effective amount of the agent are disclosed. The agent may include a clostridial neurotoxin, a fragment or a derivative thereof, attached to a targeting component, wherein the targeting component is selected from a group consisting of compounds which selectively binds at the alpha-2B or alpha-2B/alpha-2C adrenergic receptor subtype(s) as compared to other binding sites, for example, the alpha-2A adrenergic receptor subtype.

46 Claims, 1 Drawing figures

First Hit   Fwd Refs

L5: Entry 1 of 19

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NAME	CITY	STATE	ZIP CODE	COUNTRY
Gil; Daniel W.	Corona Del Mar	CA		
Aoki; Kei R.	Coto de Caza	CA		

US-CL-CURRENT: 514/1; 514/14, 514/2

## CLAIMS:

What is claimed is:

1. An agent comprising: a therapeutic component, and a targeting ligand coupled ~~coupled to the therapeutic component, the targeting ligand being effective to~~ bind to the alpha-2B or alpha-2B/alpha-.sup.2 C adrenergic receptor subtype(s). (s).

2. An agent according to claim 1 wherein the therapeutic component interferes with the release of neurotransmitters from a cell or its processes.

3. An agent according to claim 2 wherein the therapeutic component comprises a light chain component.

4. An agent according to claim 2 wherein the light chain component comprises a light chain or a fragment thereof of a botulinum toxin, a butyricum toxin, a tetani toxin or biologically active variants thereof.

5. An agent according to claim 2 wherein the light chain component comprises a light chain or a fragment thereof of a botulinum toxin type A, B, C1, D, E, F, G or biologically active variants thereof.

6. An agent according to claim 2 wherein the light chain component comprises a light chain or a fragment thereof of a botulinum toxin type A or biologically active variants thereof.

7. An agent according to claim 1 wherein the therapeutic component inactivates cellular ribosomes.

8. An agent according to claim 7 wherein the therapeutic component is saporin.

9. An agent according to claim 1 which further comprises a translocation component.

10. An agent according to claim 9 wherein the translocation component facilitates the transfer of at least a part of the agent into a cytoplasm of the target cell.
11. An agent according to claim 9 wherein the translocation component facilitates the transfer of the therapeutic component into a cytoplasm of the target cell.
12. An agent according to claim 9 wherein the translocation component comprises a heavy chain component.
13. An agent according to claim 12 wherein the heavy chain component comprises a heavy chain or a fragment thereof of a botulinum toxin, a butyricum toxin, a tetani toxin or biologically active variants thereof.
14. An agent according to claim 12 wherein the heavy chain component comprises a heavy chain or a fragment thereof of a botulinum toxin type A, B, C1, D, E, F, G or biologically active variants thereof.
15. An agent according to claim 12 wherein the heavy chain component comprises a heavy chain or a fragment thereof of a botulinum toxin type A or biologically active variants thereof.
16. An agent according to claim 15 wherein the fragment of the heavy chain comprises at least a portion of an amino terminal fragment of the heavy chain.
- 
17. An agent according to claim 9 wherein the therapeutic component comprises a light chain of a botulinum toxin type A and the translocation component comprises a fragment of a heavy chain of a botulinum toxin type A, wherein the fragment of a heavy chain can assist in the translocation of at least the therapeutic component into a cytoplasm of a cell.
18. An agent according to claim 1 wherein the targeting ligand is represented by the formula: ##STR28##
19. An agent according to claim 1 wherein the targeting ligand is a compound represented by the formula: ##STR29##
20. An agent according to claim 1 wherein the targeting ligand is a compound represented by the formula ##STR30##
21. An agent according to claim 1 wherein the targeting ligand is a compound represented by the formula: ##STR31## wherein X' is selected from the group consisting of R.sub.4 --C.dbd.C--R.sub.5 and R.sub.4 --C; a six membered carbon carbon ring structure is formed when X' is R.sub.4 --C.dbd.C--R.sub.5 ; a five membered carbon ring is formed when X' is R.sub.4 --C; R.sub.1, R.sub.2, R.sub.3, R.sub.4 and R.sub.5 are each independently selected from the group consisting of F, Cl, Br, I, OR.sub.6 and H, wherein R.sub.6 is H or an alkyl, including a methyl, an ethyl or a propyl.
22. An agent according to claim 1 wherein the targeting ligand is a compound represented by the formula: ##STR32##
23. An agent according to claim 1 wherein the targeting ligand is represented by the formula ##STR33##

wherein the dotted lines represent optional double bonds; R is H or lower alkyl; X is S or C(H)R.sub.11, wherein R.sub.11 is H or lower alkyl or R.sub.11 is absent when X is S or when the bond between X and the ring represented by ##STR34##

is a double bond; Y is O, N, S, (C(R.sub.11)X).sub.y, wherein y is an integer of from 1 to 3, --CH.dbd.CH-- or --Y.sub.1 CH.sub.2 --, wherein Y.sub.1 is O, N or S; x is an integer of 1 or 2, wherein x is 1 when R.sub.12, R.sub.13, or R.sub.14 is bound to an unsaturated carbon atom and x is 2 when R.sub.12, R.sub.13 or R.sub.14 is bonded to a saturated carbon atom; R.sub.12 is H, lower lower alkyl, halogen, hydroxy, lower alkoxy, lower alkenyl, acyl or lower alkynyl or, when attached to a saturated carbon atom, R.sub.12 may be oxo; R.sub.13 and R.sub.14 are, each, H, lower alkyl, halogen, lower alkenyl, acyl or lower alkynyl, or, when attached to a saturated carbon atom, R.sub.12 may be oxo; R.sub.13 and R.sub.14 are, each, H, lower alkyl, halogen, lower alkenyl, acyl, lower alkynyl, aryl, heteroaryl, or substituted aryl or heteroaryl, wherein said substituent is halogen, lower alkyl, lower alkoxy, lower alkenyl, acyl, lower alkynyl, nitro, cyano, trifluoromethyl, hydroxy, or phenyl or, together, are --(C(R.sub.2)x)z-; --Y.sub.1 (C(R.sub.2)x)z'-; --Y.sub.1 (C(R.sub.2)x)y Y.sub.1 -; --(C(R.sub.2)x)-Y.sub.1 --(C(R.sub.2)x)-; --(C(R.sub.2)x)- Y.sub.1 --(C(R.sub.2)x)- (C(R.sub.2)x)- and --Y.sub.1 --(C(R.sub.2)x)-Y.sub.1 --(C(R.sub.2)x)- wherein z is an integer of from 3 to 5, z' is an integer of from 2 to 4 and x and y are as defined above, and further either end of each of these divalent moieties may attach at either R.sub.13 or R.sub.14 to form the condensed ring structure ##STR35##

and the ring thus formed may be totally unsaturated, partially unsaturated, or totally saturated provided that a ring carbon has no more than 4 valences, nitrogen no more than three and O and S have no more than two.

24. An agent according to claim 1 wherein the targeting ligand comprises an amino acid component.

25. An agent according to claim 24 wherein the amino acid component is an antibody.

26. An agent according to claim 25 wherein the antibody is raised from an antigen component, the antigen component comprises a second extracellular loop of an alpha-2B receptor.

27. An agent according to claim 26 wherein the second extracellular loop is conjugated to a keyhole limpet hemocyanin.

28. An agent according to claim 24 wherein the amino acid component comprises a variant peptide, a variant polypeptide, a variant protein or a variant protein complex of a wild type peptide, polypeptide, protein or protein complex, respectively.

29. An agent according to claim 24 wherein the amino acid component is a variant polypeptide.

30. An agent according to claim 29 wherein the variant polypeptide is a variant variant heavy chain.

31. An agent according to claim 1 wherein the therapeutic component and the targeting ligand are attached to each other through a spacer component.

32. An agent according to claim 9 wherein the therapeutic component, the translocation component and the targeting ligand are attached to each other through a spacer component.

33. An agent according to claim 32 wherein the therapeutic component is a light light chain of a botulinum toxin type A, the translocation component is a fragment of a heavy chain of a botulinum toxin type A which can facilitate the translocation of at least the light chain into a cytoplasm of a cell, and the targeting component is represented by the formula: ##STR36## wherein X' is selected from the group consisting of R.sub.4 --C.dbd.C--R.sub.5 and R.sub.4 --C; a six membered carbon ring structure is formed when X' is R.sub.4 --C.dbd.C--R.sub.5 ; a five membered carbon ring is formed when X' is R.sub.4 --C; R.sub.1, R.sub.2, R.sub.3, R.sub.4 and R.sub.5 are each independently selected from the group consisting of F, Cl, Br, I, OR.sub.6 and H, wherein R.sub.6 is H or an alkyl, including a methyl, an ethyl or a propyl.

34. An agent according to claim 32 wherein the spacer component comprises a moiety selected from the group consisting of a hydrocarbon, a polypeptide other than an immunoglobulin hinge region, and a proline-containing polypeptide polypeptide identical or analogous to an immunoglobulin hinge region.

35. An agent according to claim 1 useful for treating chronic pain in a mammal, mammal, including a human.

36. An agent according to claim 35 wherein the chronic pain is treated without substantially affecting acute pain sensation or tactile sensation.

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37. A method for making an agent for treating pain comprising the step of producing a polypeptide from a gene having codes for at least one component of the agent, wherein the agent comprises a therapeutic component, and a targeting targeting ligand coupled to the therapeutic component, the targeting ligand being effective to bind to the alpha-2B or alpha-2B/alpha-2C adrenergic receptor subtype(s).

38. A method for making an agent according to claim 37 wherein the agent further comprises a translocation component.

39. A method according to claim 38 wherein the therapeutic component comprises a light chain of botulium toxin type A and the translocation component comprises a fragment of a heavy chain which is able to facilitate the transfer of at least the light chain into a cytoplasm of the target cell.

40. A method according to claim 38 wherein the targeting ligand comprises an amino acid component.

41. A method according to claim 40 wherein the amino acid component comprises a variant peptide, a variant polypeptide, a variant protein, or a variant protein complex of a wild type peptide, polypeptide, protein or protein complex, respectively.

42. A method according to claim 41 wherein the variant peptide is a variant heavy chain.

43. The agent of claim 1, wherein the targeting ligand selectively binds to the the alpha-2B or alpha-2B/alpha-2C adrenergic receptor subtype(s) as compared to to the alpha-2A adrenergic receptor subtype.

44. The agent of claim 37, wherein the targeting ligand of the agent selectively binds to the alpha-2B or alpha-2B/alpha-2C adrenergic receptor subtype(s) as compared to the alpha-2A adrenergic receptor subtype.

45. An agent comprising: a therapeutic component, and a targeting component coupled to the therapeutic component, the targeting component being represented represented by the formula: ##STR37##

46. An agent comprising: a therapeutic component, and a targeting component coupled to the therapeutic component, the targeting component comprising an antibody raised from an antigen component comprising a second extracellular loop, the second extracellular loop comprising an amino acid sequence of KGDQGPQPRGRPQCKLNQE (SEQ ID NO: 1).

## WEST Search History

DATE: Tuesday, September 07, 2004

Hide?	Set Name	Query	Hit Count
		<i>DB=USPT; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L1	5252482.pn.	1
		<i>DB=EPAB,JPAB,DWPI; PLUR=YES; OP=AND</i>	
<input type="checkbox"/>	L2	shone.in.	120
<input type="checkbox"/>	L3	L2 and \$toxin	14

END OF SEARCH HISTORY

File 155:MEDLINE(R) 1951-2004/Sep W1

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\*File 155: Medline has been reloaded. Accession numbers have changed. Please see HELP NEWS 154 for details.

Set	Items	Description
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Cost is in DialUnits

?b 155 medicine

08sep04 09:10:54 User228206 Session D2229.8

\$0.36 0.114 DialUnits File155

\$0.36 Estimated cost File155

\$0.36 Estimated cost this search

\$0.36 Estimated total session cost 0.114 DialUnits

SYSTEM:OS - DIALOG OneSearch

File 155:MEDLINE(R) 1951-2004/Sep W1

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\*File 155: Medline has been reloaded. Accession numbers have changed. Please see HELP NEWS 154 for details.

File 5:Biosis Previews(R) 1969-2004/Aug W5

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File 34:SciSearch(R) Cited Ref Sci 1990-2004/Aug W5

(c) 2004 Inst for Sci Info

File 35:Dissertation Abs Online 1861-2004/Aug

(c) 2004 ProQuest Info&Learning

File 48:SPORTDiscus 1962-2004/Aug

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File 65:Inside Conferences 1993-2004/Sep W1

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File 71:ELSEVIER BIOBASE 1994-2004/Aug W5

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File 73:EMBASE 1974-2004/Aug W5

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File 91:MANTIS(TM) 1880-2004/Aug

2001 (c) Action Potential

File 94:JICST-EPlus 1985-2004/Aug W2

(c)2004 Japan Science and Tech Corp(JST)

File 98:General Sci Abs/Full-Text 1984-2004/Jul

(c) 2004 The HW Wilson Co.

File 135:NewsRx Weekly Reports 1995-2004/Aug W5

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\*File 135: New newsletters are now added. See Help News135 for the complete list of newsletters.

File 144:Pascal 1973-2004/Aug W5

(c) 2004 INIST/CNRS

File 149:TGG Health&Wellness DB(SM) 1976-2004/Aug W3

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File 156:ToxFile 1965-2004/Sep W1

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\*File 156: ToxFile now reloaded with 2004 MeSH.

Enter Help News156 for more information.

File 159:Cancerlit 1975-2002/Oct

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\*File 159: Cancerlit ceases updating with immediate effect.

Please see HELP NEWS.

File 162:Global Health 1983-2004/Jul

(c) 2004 CAB International

File 164:Allied & Complementary Medicine 1984-2004/Sep

(c) 2004 BLHCIS

File 172:EMBASE Alert 2004/Aug W5

(c) 2004 Elsevier Science B.V.

File 266:FEDRIP 2004/Jun

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File 369:New Scientist 1994-2004/Aug W5

(c) 2004 Reed Business Information Ltd.

File 370:Science 1996-1999/Jul W3

(c) 1999 AAAS

**\*File 370: This file is closed (no updates). Use File 47 for more current information.**

File 399:CA SEARCH(R) 1967-2004/UD=14111

(c) 2004 American Chemical Society

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Alert feature enhanced for multiple files, etc. See HELP ALERT.

File 434:SciSearch(R) Cited Ref Sci 1974-1989/Dec

(c) 1998 Inst for Sci Info

File 444:New England Journal of Med. 1985-2004/Aug W5

(c) 2004 Mass. Med. Soc.

File 467:ExtraMED(tm) 2000/Dec

(c) 2001 Informania Ltd.

**\*File 467: For information about updating status please see Help News467.**

Set Items Description

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?e epitope

Ref	Items	RT	Index-term
E1	3		EPITOPCS
E2	3		EPITOPDES
E3	192638	19	*EPITOPE
E4	2		EPITOPE (PECTIN)
E5	8		EPITOPE --ADVERSE DRUG REACTION --AE
E6	27		EPITOPE --CLINICAL TRIAL --CT
E7	15		EPITOPE --DRUG ADMINISTRATION --AD
E8	48		EPITOPE --DRUG ANALYSIS --AN
E9	27		EPITOPE --DRUG COMBINATION --CB
E10	17		EPITOPE --DRUG COMPARISON --CM
E11	1		EPITOPE --DRUG CONCENTRATION --CR
E12	119		EPITOPE --DRUG DEVELOPMENT --DV

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Ref	Items	Index-term
E13	19	EPITOPE --DRUG DOSE --DO
E14	1	EPITOPE --DRUG INTERACTION --IT
E15	103	EPITOPE --DRUG THERAPY --DT
E16	12	EPITOPE --DRUG TOXICITY --TO
E17	6004	EPITOPE --ENDOGENOUS COMPOUND --EC
E18	9	EPITOPE --INTRADERMAL DRUG ADMINISTRATION --DL
E19	1	EPITOPE --INTRAGASTRIC DRUG ADMINISTRATION --I
E20	13	EPITOPE --INTRAMUSCULAR DRUG ADMINISTRATION --
E21	2	EPITOPE --INTRANASAL DRUG ADMINISTRATION
E22	12	EPITOPE --INTRANASAL DRUG ADMINISTRATION --NA
E23	16	EPITOPE --INTRAPERITONEAL DRUG ADMINISTRATION
E24	1	EPITOPE --INTRATUMORAL DRUG ADMINISTRATION

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Ref	Items	Index-term
E25	1	EPITOPE --INTRATUMORAL DRUG ADMINISTRATION --T
E26	7	EPITOPE --INTRAVENOUS DRUG ADMINISTRATION --IV
E27	3	EPITOPE --ORAL DRUG ADMINISTRATION --PO
E28	2	EPITOPE --PARENTERAL DRUG ADMINISTRATION --PA
E29	43	EPITOPE --PHARMACEUTICS --PR
E30	1	EPITOPE --PHARMACOECONOMICS --PE
E31	3	EPITOPE --PHARMACOKINETICS --PK
E32	168	EPITOPE --PHARMACOLOGY --PD
E33	1	EPITOPE --RECTAL DRUG ADMINISTRATION --RC
E34	31	EPITOPE --SUBCUTANEOUS DRUG ADMINISTRATION --S
E35	2	EPITOPE A
E36	8	EPITOPE ACCESSIBILITY

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E37	1	EPITOPE ACQUISITION
E38	1	EPITOPE ADDITION METHOD: ISOLATION/PURIFICATIO
E39	2	EPITOPE AFFINITY
E40	7	EPITOPE AFFINITY CHROMATOGRAPHY
E41	1	EPITOPE AFFINITY EPITOPE DENSITY
E42	2	EPITOPE AFFINITY-CHROMATOGRAPHY
E43	1	EPITOPE AGDR
E44	1	EPITOPE ALPHA -GALACTOSYL
E45	1	EPITOPE ALPHA GAL
E46	1	EPITOPE ALTERATION
E47	1	EPITOPE ALTERATIONS
E48	3	EPITOPE AMINO ACID SEQUENCE

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Ref	Items	Index-term
E49	1	EPITOPE AMINO ACID SEQUENCE MOLECULAR SEQUENCE
E50	1	EPITOPE AMYLASE SOMATOSTATIN

?p

Ref	Items	Index-term
E1	1	EPITOPE AMYLASE SOMATOSTATIN
E2	1	EPITOPE ANALOGS
E3	1	EPITOPE ANALOGUE
E4	1	EPITOPE ANALOGUES
E5	1	EPITOPE ANALYSES
E6	157	EPITOPE ANALYSIS
E7	1	EPITOPE ANALYSIS CYTOTOXIC ANTIBODY PRODUCTION
E8	1	EPITOPE ANALYSIS SYSTEM
E9	2	EPITOPE ANALYSIS: ANALYSIS/CHARACTERIZATION TE
E10	1	EPITOPE ANTIBODIES
E11	3	EPITOPE ANTIBODY
E12	1	EPITOPE ANTIGEN

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Ref	Items	Index-term
E13	1	EPITOPE ANTIGEN VACCINE
E14	1	EPITOPE ANTIGENICITY
E15	1	EPITOPE ANTIGENICITY LOSS
E16	1	EPITOPE ANTIGENIQUE
E17	1	EPITOPE ANTISERUM
E18	1	EPITOPE ANTIVIRAL AB BINDING IMMUNOLOGIC PROPE
E19	1	EPITOPE API
E20	7	EPITOPE APPROACH
E21	1	EPITOPE ASSIGNMENT
E22	1	EPITOPE AUTOANTIBODY
E23	1	EPITOPE AUTOREACTIVITY
E24	1	EPITOPE AVAILABILITY CHANGE

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Ref	Items	Index-term
E25	4	EPITOPE B
E26	1	EPITOPE B/ANTIBODY-A3B10 INTERFACE
E27	1	EPITOPE BIAS
E28	25	EPITOPE BINDING
E29	1	EPITOPE BINDING SITE
E30	2	EPITOPE BINDING SPECIFICITY

E31	1	EPITOPE BINDING STRUCTURE ACTIVITY RELATIONSHI
E32	1	EPITOPE BIOCHEMICAL CHARACTERIZATION
E33	1	EPITOPE BIORECOGNITION-BASED
E34	3	EPITOPE BLOCKED PANNING
E35	1	EPITOPE BLOCKING ASSAY
E36	1	EPITOPE BLOCKING EFFECTS

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Ref	Items	Index-term
E37	1	EPITOPE BLOOD TYPE PANCREATIC CANCER
E38	1	EPITOPE B105-B110
E39	2	EPITOPE C
E40	2	EPITOPE CARRIER
E41	1	EPITOPE CARRIER DECAPEPTIDE DNA BINDING AMINO
E42	1	EPITOPE CARRIERS
E43	1	EPITOPE CENTER
E44	32	EPITOPE CHARACTERIZATION
E45	1	EPITOPE CHARACTERZATION
E46	2	EPITOPE CHIMERIC ANTIGEN
E47	1	EPITOPE CLASS
E48	1	EPITOPE CLEAVAGE

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Ref	Items	Index-term
E49	1	EPITOPE CLUSTER
E50	1	EPITOPE CLUSTER I

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Ref	Items	Index-term
E1	1	EPITOPE CLUSTER I
E2	1	EPITOPE CLUSTER II
E3	2	EPITOPE CLUSTERS
E4	1	EPITOPE COATING
E5	1	EPITOPE COLOCALIZATION
E6	1	EPITOPE COMBINATION GROUPS
E7	1	EPITOPE COMMON
E8	3	EPITOPE COMMONALITY
E9	1	EPITOPE COMPARISON
E10	1	EPITOPE COMPATIBILITY
E11	2	EPITOPE COMPLEMENTARITY
E12	3	EPITOPE COMPOSITION

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Ref	Items	Index-term
E13	13	EPITOPE CONFORMATION
E14	1	EPITOPE CONFORMATION DEPENDENCE ANTIGENIC VARI
E15	1	EPITOPE CONFORMATION DEPENDENT ELEMENTS
E16	1	EPITOPE CONFORMATIONAL CHANGE
E17	1	EPITOPE CONFORMATIONAL COMPONENT
E18	1	EPITOPE CONFORMATIONAL NATURE
E19	2	EPITOPE CONFORMATIONNEL
E20	13	EPITOPE CONSERVATION
E21	1	EPITOPE CONTEXT
E22	1	EPITOPE CONTIGUOUS CHAINS
E23	2	EPITOPE CORE STRUCTURE
E24	2	EPITOPE COVERAGE

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Ref	Items	Index-term
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E25	1	EPITOPE CRITICAL CORE
E26	1	EPITOPE CROSS-LINKING
E27	4	EPITOPE CROSS-REACTIVITY
E28	1	EPITOPE CROSS-REACTIVITY-NEGATIVE
E29	1	EPITOPE CROSS-RECOGNITION
E30	1	EPITOPE CYTOPLASMIC LOCATION
E31	1	EPITOPE C85
E32	1	EPITOPE DAMAGE
E33	1	EPITOPE DATABASE
E34	3	EPITOPE DEFICIENCY
E35	1	EPITOPE DEFINED PEPTIDES
E36	9	EPITOPE DEFINITION

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Ref	Items	Index-term
E37	1	EPITOPE DEFINITION PROBLEM
E38	1	EPITOPE DEGENERACY
E39	5	EPITOPE DELINEATION
E40	6	EPITOPE DELIVERY
E41	1	EPITOPE DELIVERY SYSTEM
E42	28	EPITOPE DENSITY
E43	1	EPITOPE DEPENDENT ANTAGONISM
E44	1	EPITOPE DEPENDENT SYNERGISM
E45	1	EPITOPE DEPHOSPHORYLATION
E46	2	EPITOPE DERIVATIVES
E47	1	EPITOPE DERIVATIZATION METHOD
E48	1	EPITOPE DESIGN AND APPLICATIONS

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Ref	Items	Index-term
E49	2	EPITOPE DESTRUCTION
E50	1	EPITOPE DETECTABILITY

?p

Ref	Items	Index-term
E1	1	EPITOPE DETECTABILITY
E2	6	EPITOPE DETECTION
E3	1	EPITOPE DETECTION V-BETA CHAIN SPECIFICITY MAJ
E4	8	EPITOPE DETERMINATION
E5	2	EPITOPE DEVICE
E6	1	EPITOPE DIAGNOSIS
E7	1	EPITOPE DIFFERENCE
E8	2	EPITOPE DIFFERENCES
E9	2	EPITOPE DIFFERENTIATION
E10	1	EPITOPE DIPEN
E11	1	EPITOPE DIRECTED ANTIBODIES
E12	5	EPITOPE DISCOVERY

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?e epitope, t-cell

Ref	Items	Index-term
E1	1	EPITOPE, NEOTOPE, METATOPE, CRYPTOTOPE, MAB, P
E2	1	EPITOPE, T LYMPHOCYTE
E3	0	*EPITOPE, T-CELL
E4	3	EPITOPEAND
E5	1	EPITOPEBEARING
E6	1	EPITOPECARRIERS
E7	1	EPITOPECLUSTER
E8	1	EPITOPECONTAINING
E9	5	EPITOPED
E10	1	EPITOPEE
E11	1	EPITOPEFLRGRAYGL

E12 3 EPITOPEIN

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?s e2

S1 1 'EPITOPE, T LYMPHOCYTE'

?s epitope?

S2 336654 EPITOPE?

?ds

Set Items Description

S1 1 'EPITOPE, T LYMPHOCYTE'

S2 336654 EPITOPE?

?e digest

Ref	Items	Index-term
E1	1	DIGESSTIBILITY
E2	1	DIGESSTION
E3	41929	*DIGEST
E4	1	DIGEST ANALYSIS
E5	1	DIGEST CANCER
E6	1	DIGEST CELLS
E7	8	DIGEST CELLULOSE
E8	1	DIGEST COLLECTION
E9	3	DIGEST CONDITIONS
E10	1	DIGEST LIQUID PHASE RETENTION TIME
E11	1	DIGEST MERZ
E12	1	DIGEST MERZ FORTE

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?s digest?

S3 2472596 DIGEST?

?e botulinum toxin

Ref	Items	RT	Index-term
E1	1		BOTULINUM TETRAVALENT (A,B,E,F) TOXOID VACCINE
E2	2		BOTULINUM TOXICITY
E3	6652	10	*BOTULINUM TOXIN
E4	1		BOTULINUM TOXIN (BOTOX)
E5	1		BOTULINUM TOXIN (THERAPEUTIC USE)
E6	336		BOTULINUM TOXIN --ADVERSE DRUG REACTION --AE
E7	220		BOTULINUM TOXIN --CLINICAL TRIAL --CT
E8	157		BOTULINUM TOXIN --DRUG ADMINISTRATION --AD
E9	15		BOTULINUM TOXIN --DRUG ANALYSIS --AN
E10	22		BOTULINUM TOXIN --DRUG COMBINATION --CB
E11	64		BOTULINUM TOXIN --DRUG COMPARISON --CM
E12	3		BOTULINUM TOXIN --DRUG CONCENTRATION --CR

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?e e3

Ref	Items	Type	RT	Index-term
R1	3701		10	*BOTULINUM TOXIN
R2	38723			DC=D5.80.90.80
R3	3761	B	56	BACTERIAL TOXIN
R4	0	S	1	BOTULINAL TOXIN TEST
R5	0	S	1	BOTULINIUM TOXIN
R6	0	S	1	BOTULINUM NEUROTOXIN
R7	0	S	1	BOTULINUM TOXINS
R8	0	S	1	BOTULINUS TOXIN
R9	0	S	1	BOTULISM TOXIN
R10	0	S	1	CLOSTRIDIUM BOTULINUM EXOTOXIN
R11	0	S	1	CLOSTRIDIUM BOTULINUM TOXIN

?e botulinum toxin

Ref	Items	RT	Index-term
E1	1		BOTULINUM TETRAVALENT (A,B,E,F) TOXOID VACCINE
E2	2		BOTULINUM TOXICITY

E3	6652	10	*BOTULINUM TOXIN
E4	1		BOTULINUM TOXIN (BOTOX)
E5	1		BOTULINUM TOXIN (THERAPEUTIC USE)
E6	336		BOTULINUM TOXIN --ADVERSE DRUG REACTION --AE
E7	220		BOTULINUM TOXIN --CLINICAL TRIAL --CT
E8	157		BOTULINUM TOXIN --DRUG ADMINISTRATION --AD
E9	15		BOTULINUM TOXIN --DRUG ANALYSIS --AN
E10	22		BOTULINUM TOXIN --DRUG COMBINATION --CB
E11	64		BOTULINUM TOXIN --DRUG COMPARISON --CM
E12	3		BOTULINUM TOXIN --DRUG CONCENTRATION --CR

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Ref	Items	Index-term
E13	28	BOTULINUM TOXIN --DRUG DEVELOPMENT --DV
E14	223	BOTULINUM TOXIN --DRUG DOSE --DO
E15	19	BOTULINUM TOXIN --DRUG INTERACTION --IT
E16	1803	BOTULINUM TOXIN --DRUG THERAPY --DT
E17	285	BOTULINUM TOXIN --DRUG TOXICITY --TO
E18	101	BOTULINUM TOXIN --ENDOGENOUS COMPOUND --EC
E19	1	BOTULINUM TOXIN --EPIDURAL DRUG ADMINISTRATION
E20	21	BOTULINUM TOXIN --INTRADERMAL DRUG ADMINISTRATION
E21	2	BOTULINUM TOXIN --INTRADUODENAL DRUG ADMINISTRATION
E22	4	BOTULINUM TOXIN --INTRAGASTRIC DRUG ADMINISTRATION
E23	7	BOTULINUM TOXIN --INTRALESIONAL DRUG ADMINISTRATION
E24	157	BOTULINUM TOXIN --INTRAMUSCULAR DRUG ADMINISTRATION

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Ref	Items	Index-term
E25	2	BOTULINUM TOXIN --INTRAOCULAR DRUG ADMINISTRATION
E26	8	BOTULINUM TOXIN --INTRAPERITONEAL DRUG ADMINISTRATION
E27	2	BOTULINUM TOXIN --INTRATHECAL DRUG ADMINISTRATION
E28	2	BOTULINUM TOXIN --INTRAURETHRAL DRUG ADMINISTRATION
E29	1	BOTULINUM TOXIN --INTRAVENOUS DRUG ADMINISTRATION
E30	11	BOTULINUM TOXIN --INTRAVESICAL DRUG ADMINISTRATION
E31	4	BOTULINUM TOXIN --ORAL DRUG ADMINISTRATION --P
E32	1	BOTULINUM TOXIN --PERIOcular DRUG ADMINISTRATION
E33	41	BOTULINUM TOXIN --PHARMACEUTICS --PR
E34	39	BOTULINUM TOXIN --PHARMACOECONOMICS --PE
E35	19	BOTULINUM TOXIN --PHARMACOKINETICS --PK
E36	416	BOTULINUM TOXIN --PHARMACOLOGY --PD

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Ref	Items	RT	Index-term
E37	2		BOTULINUM TOXIN --RECTAL DRUG ADMINISTRATION --
E38	2		BOTULINUM TOXIN --REGIONAL PERFUSION --RP
E39	2		BOTULINUM TOXIN --RETROBULBAR DRUG ADMINISTRATION
E40	2		BOTULINUM TOXIN --SUBCONJUNCTIVAL DRUG ADMINISTRATION
E41	18		BOTULINUM TOXIN --SUBCUTANEOUS DRUG ADMINISTRATION
E42	1		BOTULINUM TOXIN --TOPICAL DRUG ADMINISTRATION
E43	1		BOTULINUM TOXIN --TRANSDERMAL DRUG ADMINISTRATION
E44	3674	20	BOTULINUM TOXIN A
E45	1		BOTULINUM TOXIN A (BTX-A)
E46	730		BOTULINUM TOXIN A --ADVERSE DRUG REACTION --AE
E47	517		BOTULINUM TOXIN A --CLINICAL TRIAL --CT
E48	284		BOTULINUM TOXIN A --DRUG ADMINISTRATION --AD

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Ref	Items	Index-term
E49	16	BOTULINUM TOXIN A --DRUG ANALYSIS --AN

E50 30 BOTULINUM TOXIN A --DRUG COMBINATION --CB  
?p

Ref	Items	Index-term
E1	30	BOTULINUM TOXIN A --DRUG COMBINATION --CB
E2	137	BOTULINUM TOXIN A --DRUG COMPARISON --CM
E3	3	BOTULINUM TOXIN A --DRUG CONCENTRATION --CR
E4	32	BOTULINUM TOXIN A --DRUG DEVELOPMENT --DV
E5	603	BOTULINUM TOXIN A --DRUG DOSE --DO
E6	23	BOTULINUM TOXIN A --DRUG INTERACTION --IT
E7	2091	BOTULINUM TOXIN A --DRUG THERAPY --DT
E8	129	BOTULINUM TOXIN A --DRUG TOXICITY --TO
E9	47	BOTULINUM TOXIN A --ENDOGENOUS COMPOUND --EC
E10	2	BOTULINUM TOXIN A --INTRACEREBRAL DRUG ADMINIS
E11	82	BOTULINUM TOXIN A --INTRADERMAL DRUG ADMINISTR
E12	1	BOTULINUM TOXIN A --INTRADUODENAL DRUG ADMINIS

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Ref	Items	Index-term
E13	3	BOTULINUM TOXIN A --INTRAGASTRIC DRUG ADMINIST
E14	12	BOTULINUM TOXIN A --INTRALESIONAL DRUG ADMINIS
E15	467	BOTULINUM TOXIN A --INTRAMUSCULAR DRUG ADMINIS
E16	2	BOTULINUM TOXIN A --INTRANASAL DRUG ADMINISTRA
E17	4	BOTULINUM TOXIN A --INTRAOCULAR DRUG ADMINISTR
E18	4	BOTULINUM TOXIN A --INTRAPERITONEAL DRUG ADMIN
E19	1	BOTULINUM TOXIN A --INTRASPINAL DRUG ADMINISTR
E20	8	BOTULINUM TOXIN A --INTRAURETHRAL DRUG ADMINIS
E21	3	BOTULINUM TOXIN A --INTRAVENOUS DRUG ADMINISTR
E22	8	BOTULINUM TOXIN A --INTRAVERSICAL DRUG ADMINIST
E23	1	BOTULINUM TOXIN A --INTRAVITREAL DRUG ADMINIST
E24	2	BOTULINUM TOXIN A --ORAL DRUG ADMINISTRATION -

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?p

Ref	Items	Index-term
E25	6	BOTULINUM TOXIN A --PERIOcular DRUG ADMINISTRA
E26	99	BOTULINUM TOXIN A --PHARMACEUTICS --PR
E27	75	BOTULINUM TOXIN A --PHARMACOECONOMICS --PE
E28	36	BOTULINUM TOXIN A --PHARMACOKINETICS --PK
E29	715	BOTULINUM TOXIN A --PHARMACOLOGY --PD
E30	3	BOTULINUM TOXIN A --RECTAL DRUG ADMINISTRATION
E31	2	BOTULINUM TOXIN A --REGIONAL PERFUSION --RP
E32	2	BOTULINUM TOXIN A --RETROBULBAR DRUG ADMINISTR
E33	1	BOTULINUM TOXIN A --SUBCONJUNCTIVAL DRUG ADMIN
E34	57	BOTULINUM TOXIN A --SUBCUTANEOUS DRUG ADMINIST
E35	4	BOTULINUM TOXIN A --TOPICAL DRUG ADMINISTRATION
E36	6	BOTULINUM TOXIN A --TRANSDERMAL DRUG ADMINISTR

Enter P or PAGE for more

?e botulinum toxin b

Ref	Items	RT	Index-term
E1	1		BOTULINUM TOXIN ASSAY
E2	1		BOTULINUM TOXIN AUTONOMIC AGENT CENTRAL DEPRES
E3	490	8	*BOTULINUM TOXIN B
E4	124		BOTULINUM TOXIN B --ADVERSE DRUG REACTION --AE
E5	70		BOTULINUM TOXIN B --CLINICAL TRIAL --CT
E6	20		BOTULINUM TOXIN B --DRUG ADMINISTRATION --AD
E7	8		BOTULINUM TOXIN B --DRUG ANALYSIS --AN
E8	4		BOTULINUM TOXIN B --DRUG COMBINATION --CB
E9	71		BOTULINUM TOXIN B --DRUG COMPARISON --CM
E10	5		BOTULINUM TOXIN B --DRUG DEVELOPMENT --DV
E11	105		BOTULINUM TOXIN B --DRUG DOSE --DO
E12	4		BOTULINUM TOXIN B --DRUG INTERACTION --IT

Enter P or PAGE for more

?p

Ref	Items	Index-term
E13	216	BOTULINUM TOXIN B --DRUG THERAPY --DT
E14	46	BOTULINUM TOXIN B --DRUG TOXICITY --TO
E15	24	BOTULINUM TOXIN B --ENDOGENOUS COMPOUND --EC
E16	1	BOTULINUM TOXIN B --INHALATIONAL DRUG ADMINIST
E17	4	BOTULINUM TOXIN B --INTRADERMAL DRUG ADMINISTR
E18	2	BOTULINUM TOXIN B --INTRALESIONAL DRUG ADMINIS
E19	78	BOTULINUM TOXIN B --INTRAMUSCULAR DRUG ADMINIS
E20	2	BOTULINUM TOXIN B --INTRAPERITONEAL DRUG ADMIN
E21	2	BOTULINUM TOXIN B --INTRAVESICAL DRUG ADMINIST
E22	37	BOTULINUM TOXIN B --PHARMACEUTICS --PR
E23	16	BOTULINUM TOXIN B --PHARMACOECONOMICS --PE
E24	7	BOTULINUM TOXIN B --PHARMACOKINETICS --PK

Enter P or PAGE for more

?p

Ref	Items	Index-term
E25	118	BOTULINUM TOXIN B --PHARMACOLOGY --PD
E26	3	BOTULINUM TOXIN B --SUBCUTANEOUS DRUG ADMINIST
E27	1	BOTULINUM TOXIN B ANTIBODY
E28	1	BOTULINUM TOXIN B INHIBITOR
E29	1	BOTULINUM TOXIN B MYOBLOC
E30	1	BOTULINUM TOXIN B TYPE
E31	1	BOTULINUM TOXIN B.
E32	1	BOTULINUM TOXIN BINDING SITES
E33	1	BOTULINUM TOXIN BONT
E34	29	BOTULINUM TOXIN BOTOX
E35	2	BOTULINUM TOXIN BOTOX DYSPORT
E36	1	BOTULINUM TOXIN BOTOX DYSPORT NEUROBLOC

Enter P or PAGE for more

?s e3-e31

490	BOTULINUM TOXIN B
124	BOTULINUM TOXIN B --ADVERSE DRUG REACTION --AE
70	BOTULINUM TOXIN B --CLINICAL TRIAL --CT
20	BOTULINUM TOXIN B --DRUG ADMINISTRATION --AD
8	BOTULINUM TOXIN B --DRUG ANALYSIS --AN
4	BOTULINUM TOXIN B --DRUG COMBINATION --CB
71	BOTULINUM TOXIN B --DRUG COMPARISON --CM
5	BOTULINUM TOXIN B --DRUG DEVELOPMENT --DV
105	BOTULINUM TOXIN B --DRUG DOSE --DO
4	BOTULINUM TOXIN B --DRUG INTERACTION --IT
216	BOTULINUM TOXIN B --DRUG THERAPY --DT
46	BOTULINUM TOXIN B --DRUG TOXICITY --TO
24	BOTULINUM TOXIN B --ENDOGENOUS COMPOUND --EC
1	BOTULINUM TOXIN B --INHALATIONAL DRUG ADMINIST
4	BOTULINUM TOXIN B --INTRADERMAL DRUG ADMINISTR
2	BOTULINUM TOXIN B --INTRALESIONAL DRUG ADMINIS
78	BOTULINUM TOXIN B --INTRAMUSCULAR DRUG ADMINIS
2	BOTULINUM TOXIN B --INTRAPERITONEAL DRUG ADMIN
2	BOTULINUM TOXIN B --INTRAVESICAL DRUG ADMINIST
37	BOTULINUM TOXIN B --PHARMACEUTICS --PR
16	BOTULINUM TOXIN B --PHARMACOECONOMICS --PE
7	BOTULINUM TOXIN B --PHARMACOKINETICS --PK
118	BOTULINUM TOXIN B --PHARMACOLOGY --PD
3	BOTULINUM TOXIN B --SUBCUTANEOUS DRUG ADMINIST
1	BOTULINUM TOXIN B ANTIBODY
1	BOTULINUM TOXIN B INHIBITOR
1	BOTULINUM TOXIN B MYOBLOC
1	BOTULINUM TOXIN B TYPE
1	BOTULINUM TOXIN B.
S4	493 E3-E31

?p

Ref	Items	Index-term
E37	4	BOTULINUM TOXIN BTX
E38	1	BOTULINUM TOXIN BTX-A
E39	1	BOTULINUM TOXIN BTX-A DYSPORT
E40	57	BOTULINUM TOXIN C
E41	1	BOTULINUM TOXIN C TREATMENT
E42	30	BOTULINUM TOXIN C 2
E43	1	BOTULINUM TOXIN C 2 ANTIBODY
E44	1	BOTULINUM TOXIN CHEMODENERVATION
E45	1	BOTULINUM TOXIN CHOLERA TOXIN GTP-BINDING PROT
E46	5	BOTULINUM TOXIN COMPLEX
E47	27	BOTULINUM TOXIN C1
E48	1	BOTULINUM TOXIN C1 LIGHT CHAIN

Enter P or PAGE for more

?p

Ref	Items	Index-term
E49	14	BOTULINUM TOXIN C3
E50	40	BOTULINUM TOXIN D

?s e40-e43 or e47-e50

57	BOTULINUM TOXIN C
1	BOTULINUM TOXIN C TREATMENT
30	BOTULINUM TOXIN C 2
1	BOTULINUM TOXIN C 2 ANTIBODY
27	BOTULINUM TOXIN C1
1	BOTULINUM TOXIN C1 LIGHT CHAIN
14	BOTULINUM TOXIN C3
40	BOTULINUM TOXIN D

s5 136 E40-E43 OR E47-E50

?p

Ref	Items	RT	Index-term
E1	40		BOTULINUM TOXIN D
E2	1		BOTULINUM TOXIN D LIGHT CHAIN
E3	1		BOTULINUM TOXIN DERIVATIVE
E4	2		BOTULINUM TOXIN DETECTION
E5	7		BOTULINUM TOXIN DYSPORT
E6	85	5	BOTULINUM TOXIN E
E7	1		BOTULINUM TOXIN E --CLINICAL TRIAL --CT
E8	2		BOTULINUM TOXIN E --DRUG ADMINISTRATION --AD
E9	1		BOTULINUM TOXIN E --DRUG ANALYSIS --AN
E10	3		BOTULINUM TOXIN E --DRUG COMPARISON --CM
E11	1		BOTULINUM TOXIN E --DRUG DOSE --DO
E12	3		BOTULINUM TOXIN E --DRUG THERAPY --DT

Enter P or PAGE for more

?p

Ref	Items	Index-term
E13	1	BOTULINUM TOXIN E --ENDOGENOUS COMPOUND --EC
E14	2	BOTULINUM TOXIN E --INTRAMUSCULAR DRUG ADMINIS
E15	1	BOTULINUM TOXIN E --PHARMACEUTICS --PR
E16	7	BOTULINUM TOXIN E --PHARMACOLOGY --PD
E17	1	BOTULINUM TOXIN E BONT/E
E18	1	BOTULINUM TOXIN E LIGHT CHAIN
E19	1	BOTULINUM TOXIN EVANESCENT WAVE IMMUNOASSAY OP
E20	1	BOTULINUM TOXIN EXOENZYME C3
E21	47	BOTULINUM TOXIN F
E22	1	BOTULINUM TOXIN FORMATION
E23	2	BOTULINUM TOXIN FORMULATION
E24	12	BOTULINUM TOXIN G

Enter P or PAGE for more

?s e1-e24

40 BOTULINUM TOXIN D  
 1 BOTULINUM TOXIN D LIGHT CHAIN  
 1 BOTULINUM TOXIN DERIVATIVE  
 2 BOTULINUM TOXIN DETECTION  
 7 BOTULINUM TOXIN DYSPORT  
 85 BOTULINUM TOXIN E  
 1 BOTULINUM TOXIN E --CLINICAL TRIAL --CT  
 2 BOTULINUM TOXIN E --DRUG ADMINISTRATION --AD  
 1 BOTULINUM TOXIN E --DRUG ANALYSIS --AN  
 3 BOTULINUM TOXIN E --DRUG COMPARISON --CM  
 1 BOTULINUM TOXIN E --DRUG DOSE --DO  
 3 BOTULINUM TOXIN E --DRUG THERAPY --DT  
 1 BOTULINUM TOXIN E --ENDOGENOUS COMPOUND --EC  
 2 BOTULINUM TOXIN E --INTRAMUSCULAR DRUG ADMINIS  
 1 BOTULINUM TOXIN E --PHARMACEUTICS --PR  
 7 BOTULINUM TOXIN E --PHARMACOLOGY --PD  
 1 BOTULINUM TOXIN E BONT/E  
 1 BOTULINUM TOXIN E LIGHT CHAIN  
 1 BOTULINUM TOXIN EVANESCENT WAVE IMMUNOASSAY OP  
 1 BOTULINUM TOXIN EXOENZYME C3  
 47 BOTULINUM TOXIN F  
 1 BOTULINUM TOXIN FORMATION  
 2 BOTULINUM TOXIN FORMULATION  
 12 BOTULINUM TOXIN G

S6 154 E1-E24

?p

Ref	Items	Index-term
E25	2	BOTULINUM TOXIN GENE
E26	1	BOTULINUM TOXIN GLUTEUS MAXIMUS MUSCLES SOLEUS
E27	2	BOTULINUM TOXIN HEAVY CHAIN
E28	1	BOTULINUM TOXIN HEAVY CHAIN COMPONENT
E29	1	BOTULINUM TOXIN HISTORICAL DEVELOPMENTS
E30	11	BOTULINUM TOXIN I 125
E31	1	BOTULINUM TOXIN IMPLANT
E32	1	BOTULINUM TOXIN IN FISH
E33	1	BOTULINUM TOXIN IN VITRO ASSAY
E34	1	BOTULINUM TOXIN INHIBITOR
E35	76	BOTULINUM TOXIN INJECTION
E36	1	BOTULINUM TOXIN INJECTION AMPLIFIER

Enter P or PAGE for more

?s e28

S7 1 'BOTULINUM TOXIN HEAVY CHAIN COMPONENT'

?ds

Set	Items	Description
S1	1	'EPITOPE, T LYMPHOCYTE'
S2	336654	EPITOPE?
S3	2472596	DIGEST?
S4	493	E3-E31
S5	136	E40-E43 OR E47-E50
S6	154	E1-E24
S7	1	'BOTULINUM TOXIN HEAVY CHAIN COMPONENT'
?s (s1 or s2 or s3) and (s4 or s5 or s6 or s7)		
	1	S1
	336654	S2
	2472596	S3
	493	S4
	136	S5
	154	S6
	1	S7
S8	23	(S1 OR S2 OR S3) AND (S4 OR S5 OR S6 OR S7)

?rd

...completed examining records

S9 22 RD (unique items)

?s s9/1996:2004

```
Processing
Processed 10 of 26 files ...
>>>One or more prefixes are unsupported
>>> or undefined in one or more files.
>>>Year ranges not supported in one or more files
Completed processing all files
      22 S9
      43495488 PY=1996 : PY=2004
      S10      19 S9/1996:2004
?s s9 not s10
      22 S9
      19 S10
      S11      3 S9 NOT S10
?t s11/6/all
```

File 155:MEDLINE(R) 1951-2004/Sep W1

(c) format only 2004 The Dialog Corp.

**\*File 155: Medline has been reloaded. Accession numbers**  
have changed. Please see HELP NEWS 154 for details.

Set Items Description  
--- ----

?e t cell

Ref	Items	Index-term
E1	1	T BETA SUB 4
E2	1	T BETA 4
E3	0	*T CELL
E4	48	T CELL FACTOR 3
E5	122	T CELL FACTOR 4
E6	10	T CELL RECEPTOR INTERACTING MOLECULE
E7	8	T CELL RECEPTOR PEPTIDE VBETA5.2
E8	9	T CELL RECEPTOR PEPTIDE VBETA8.1
E9	1	T CELL RECEPTOR VALPHA28
E10	6	T CELL RECEPTOR VBETA6.7A
E11	33	T CELL RECEPTOR VBETA8
E12	8	T CELL RECEPTOR-V(BETA)8-39-59 PEPTIDE

Enter P or PAGE for more

?p

Ref	Items	RT	Index-term
E13	3		T CELL-ACTIVATING LYMPHOKINE
E14	0	1	T CHAIN
E15	164		T COMPLEX PROTEINS
E16	1		T HOFF JH VAN
E17	5		T HOLIN, BACTERIOPHAGE T4
E18	1		T LYMPHOCYTE ACTIVATION MOLECULE H4
E19	7		T LYMPHOCYTE EARLY-ACTIVATION PROTEIN
E20	11		T LYMPHOCYTE TRIGGERING FACTOR
E21	0	1	T RNA METHYLTRANSFERASES
E22	1		T SUB R ALPHA
E23	1		T SUB 3 R ALPHA
E24	1		T SUB 3 R SUB BETA

Enter P or PAGE for more

?p

Ref	Items	RT	Index-term
E25	5		T SUP HP
E26	1		T SUP WL
E27	2		T SUP W1
E28	1		T SUP W12
E29	1		T SUP W124
E30	3		T SUP W18
E31	2		T SUP W5
E32	1		T SUP W73
E33	2		T SUP 12
E34	2		T V. T
E35	0	1	T VIRUS
E36	1		T 0070907

Enter P or PAGE for more

?p

Ref	Items	Index-term
E37	4	T 0128
E38	1	T 0162
E39	20	T 0201
E40	2	T 0314407
E41	5	T 0632
E42	2	T 0757

E43	29	T 0901317
E44	3	T 0970
E45	9	T 1032
E46	15	T 107
E47	11	T 1095
E48	1	T 113242

Enter P or PAGE for more

?p

Ref	Items	Index-term
E49	3	T 138067
E50	7	T 1583

?p

Ref	Items	Index-term
E1	7	T 1583
E2	2	T 1815
E3	1	T 2153
E4	2	T 22083
E5	1	T 241
E6	2	T 2585
E7	1	T 2591
E8	14	T 30177
E9	1	T 30695
E10	2	T 330
E11	7	T 3762
E12	5	T 3811ME

Enter P or PAGE for more

?e t

Ref	Items	RT	Index-term
E1	3		S9947
E2	3		S9977
E3	3724719		*T
E4	1762		T / (TROPONIN T)
E5	0		T //DOCUME (VALIDATION STUDIES [PUBLICATION TYPE])
E6	117		T //PE (PEPTIDE T)
E7	402		T //POLY (POLY T)
E8	5		T ANTIGEN, MUCIN-TYPE CARBOHYDRATE
E9	0	1	T ANTIGENS
E10	1		T BERNHARD
E11	1		T BETA -I
E12	1		T BETA -II

Enter P or PAGE for more

?s e3

S1 3724719 'T'

?s s1 (2n) cell?

3724719 S1  
2535888 CELL?

S2 177843 S1 (2N) CELL?

?e clostridium botulinum

Ref	Items	RT	Index-term
E1	2		CLOSTRIDIUM BARATI F TOXIN
E2	1		CLOSTRIDIUM BIFERMENTANS
E3	1888	6	*CLOSTRIDIUM BOTULINUM
E4	73		CLOSTRIDIUM BOTULINUM --ANALYSIS --AN
E5	45		CLOSTRIDIUM BOTULINUM --CHEMISTRY --CH
E6	129		CLOSTRIDIUM BOTULINUM --CLASSIFICATION --CL
E7	23		CLOSTRIDIUM BOTULINUM --CYTOLOGY --CY
E8	106		CLOSTRIDIUM BOTULINUM --DRUG EFFECTS --DE
E9	125		CLOSTRIDIUM BOTULINUM --ENZYMولوجY --EN
E10	136		CLOSTRIDIUM BOTULINUM --GENETICS --GE
E11	244		CLOSTRIDIUM BOTULINUM --GROWTH AND DEVELOPMENT

E12 188 CLOSTRIDIUM BOTULINUM --IMMUNOLOGY --IM

Enter P or PAGE for more

?p

Ref	Items	RT	Index-term
E13	449		CLOSTRIDIUM BOTULINUM --ISOLATION AND PURIFICA
E14	303		CLOSTRIDIUM BOTULINUM --METABOLISM --ME
E15	113		CLOSTRIDIUM BOTULINUM --PATHOGENICITY --PY
E16	69		CLOSTRIDIUM BOTULINUM --PHYSIOLOGY --PH
E17	51		CLOSTRIDIUM BOTULINUM --RADIATION EFFECTS --RE
E18	14		CLOSTRIDIUM BOTULINUM --ULTRASTRUCTURE --UL
E19	1		CLOSTRIDIUM BOTULINUM --VIROLOGY --VI
E20	2		CLOSTRIDIUM BOTULINUM CRYOPROTEIN
E21	4		CLOSTRIDIUM BOTULINUM L TOXIN
E22	1		CLOSTRIDIUM BOTULINUM LL TOXIN
E23	6		CLOSTRIDIUM BOTULINUM M TOXIN
E24	0	1	CLOSTRIDIUM BOTULINUM TOXINS

Enter P or PAGE for more

?p

Ref	Items	RT	Index-term
E25	29		CLOSTRIDIUM BOTULINUM TOXOID
E26	1		CLOSTRIDIUM BOTULINUM/CHEMISTRY
E27	14		CLOSTRIDIUM BOTULINUM/CULTURE
E28	1		CLOSTRIDIUM BOTULINUM/EFFECT OF DRUGS ON
E29	1		CLOSTRIDIUM BOTULINUM/EFFECT OF RADIATIONS ON
E30	10		CLOSTRIDIUM BOTULINUM/IMMUNOLOGY
E31	2		CLOSTRIDIUM BOTULINUM/METABOLISM
E32	3		CLOSTRIDIUM BOTULINUM/PHARMACOLOGY
E33	1		CLOSTRIDIUM BOTULINUM/RADIATION EFFECTS
E34	4		CLOSTRIDIUM BUTYRICUM
E35	1		CLOSTRIDIUM CHAUVOEI
E36	1524	6	CLOSTRIDIUM DIFFICILE

Enter P or PAGE for more

?s e3-e33

1888	CLOSTRIDIUM BOTULINUM
73	CLOSTRIDIUM BOTULINUM --ANALYSIS --AN
45	CLOSTRIDIUM BOTULINUM --CHEMISTRY --CH
129	CLOSTRIDIUM BOTULINUM --CLASSIFICATION --CL
23	CLOSTRIDIUM BOTULINUM --CYTOLOGY --CY
106	CLOSTRIDIUM BOTULINUM --DRUG EFFECTS --DE
125	CLOSTRIDIUM BOTULINUM --ENZYMOLOGY --EN
136	CLOSTRIDIUM BOTULINUM --GENETICS --GE
244	CLOSTRIDIUM BOTULINUM --GROWTH AND DEVELOPMENT
188	CLOSTRIDIUM BOTULINUM --IMMUNOLOGY --IM
449	CLOSTRIDIUM BOTULINUM --ISOLATION AND PURIFICA
303	CLOSTRIDIUM BOTULINUM --METABOLISM --ME
113	CLOSTRIDIUM BOTULINUM --PATHOGENICITY --PY
69	CLOSTRIDIUM BOTULINUM --PHYSIOLOGY --PH
51	CLOSTRIDIUM BOTULINUM --RADIATION EFFECTS --RE
14	CLOSTRIDIUM BOTULINUM --ULTRASTRUCTURE --UL
1	CLOSTRIDIUM BOTULINUM --VIROLOGY --VI
2	CLOSTRIDIUM BOTULINUM CRYOPROTEIN
4	CLOSTRIDIUM BOTULINUM L TOXIN
1	CLOSTRIDIUM BOTULINUM LL TOXIN
6	CLOSTRIDIUM BOTULINUM M TOXIN
0	CLOSTRIDIUM BOTULINUM TOXINS
29	CLOSTRIDIUM BOTULINUM TOXOID
1	CLOSTRIDIUM BOTULINUM/CHEMISTRY
14	CLOSTRIDIUM BOTULINUM/CULTURE
1	CLOSTRIDIUM BOTULINUM/EFFECT OF DRUGS ON
1	CLOSTRIDIUM BOTULINUM/EFFECT OF RADIATIONS ON
10	CLOSTRIDIUM BOTULINUM/IMMUNOLOGY
2	CLOSTRIDIUM BOTULINUM/METABOLISM

3 CLOSTRIDIUM BOTULINUM/PHARMACOLOGY  
 1 CLOSTRIDIUM BOTULINUM/RADIATION EFFECTS  
 S3 1932 E3-E33

?e e24

Ref	Items	Type	RT	Index-term
R1	0		1	*CLOSTRIDIUM BOTULINUM TOXINS
R2	4166	X	11	BOTULINUM TOXINS

?s r1-r2

0 CLOSTRIDIUM BOTULINUM TOXINS  
 4166 BOTULINUM TOXINS

S4 4166 R1-R2

?e r2

Ref	Items	Type	RT	Index-term
R1	4166		11	*BOTULINUM TOXINS
R2	4166	X		DC=D24.185.926.123.179. (BOTULINUM TOXINS)
R3	4166	X		DC=D24.185.926.640.75. (BOTULINUM TOXINS)
R4	170	X	1	BOTULIN
R5	0	X	1	CLOSTRIDIUM BOTULINUM TOXINS
R6	2329	R	10	BOTULISM
R7	882	R	109	CHOLINERGIC AGENTS
R8	1888	R	6	CLOSTRIDIUM BOTULINUM
R9	545	B	29	ANTI-DYSKINESIA AGENTS
R10	12046	B	17	BACTERIAL TOXINS
R11	8982	B	15	NEUROTOXINS
R12	1502	N	4	BOTULINUM TOXIN TYPE A

?p

>>>Related terms display completed...

?s r1-r12

4166 BOTULINUM TOXINS  
 4166 DC=D24.185.926.123.179. (BOTULINUM TOXINS)  
 4166 DC=D24.185.926.640.75. (BOTULINUM TOXINS)

170 BOTULIN  
 0 CLOSTRIDIUM BOTULINUM TOXINS  
 2329 BOTULISM  
 882 CHOLINERGIC AGENTS  
 1888 CLOSTRIDIUM BOTULINUM  
 545 ANTI-DYSKINESIA AGENTS  
 12046 BACTERIAL TOXINS  
 8982 NEUROTOXINS  
 1502 BOTULINUM TOXIN TYPE A

S5 29409 R1-R12

?ds

Set	Items	Description
S1	3724719	'T'
S2	177843	S1 (2N) CELL?
S3	1932	E3-E33
S4	4166	R1-R2
S5	29409	R1-R12

?s s2 and (s3 or s4 or s5)

177843 S2  
 1932 S3  
 4166 S4  
 29409 S5

S6 388 S2 AND (S3 OR S4 OR S5)

?s s6/1995:2004

388 S6  
 4638017 PY=1995 : PY=2004

S7 255 S6/1995:2004

?s s6 not s7

388 S6  
 255 S7

S8 133 S6 NOT S7

?s s8 and (epitop? or peptid? or polypeptid? or sequenc?)

133 S8

77352 EPITOP?  
 376415 PEPTID?  
 84600 POLYPEPTID?  
 716920 SEQUENC?  
 S9 47 S8 AND (EPITOP? OR PEPTID? OR POLYPEPTID? OR SEQUENC?)  
 ?s s9 and light? and heavy?  
 47 S9  
 268406 LIGHT?  
 64519 HEAVY?  
 S10 3 S9 AND LIGHT? AND HEAVY?  
 ?s s8 and terminal? and epitop?  
 133 S8  
 248111 TERMINAL?  
 77352 EPITOP?  
 S11 1 S8 AND TERMINAL? AND EPITOP?  
 ?s s8 and light? and heavy?  
 133 S8  
 268406 LIGHT?  
 64519 HEAVY?  
 S12 4 S8 AND LIGHT? AND HEAVY?  
 ?s s10 or s11 or s12  
 3 S10  
 1 S11  
 4 S12  
 S13 5 S10 OR S11 OR S12  
 ?t s13/9/all

12907494 PMID: 8577267

**Immunological characterization of the neurotoxin produced by Clostridium botulinum type A associated with infant botulism in Japan.**

Kozaki S; Nakane S; Kamata Y

Department of Veterinary Science, College of Agriculture, University of Osaka Prefecture, Japan.

Microbiology and immunology (JAPAN) 1995, 39 (10) p767-74, ISSN 0385-5600 Journal Code: 7703966

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The neurotoxin associated with type A infant **botulism** in Japan shows different antigenic properties from those produced by authentic strains. The monoclonal antibodies recognizing the light chain reacted to both neurotoxins, whereas half the antibodies recognizing the heavy chain reacted specifically to the respective neurotoxin. Each neurotoxin showed its own manner of binding to brain synaptosomes. These results indicate that the distinguishable characteristics are ascribable to the heavy chain but not to the light chain. In both neurotoxins, an **epitope** recognized by the monoclonal antibody that reacts to the light chain and neutralizes the toxin was found to be very close to the amino-terminal half (H-1 fragment) of the heavy chain. This may support the hypothesis that the H-1 fragment functions in the transport of the light chain in the target cell.

Tags: Human

Descriptors: **Botulinum** Toxins--chemistry--CH; \* **Botulism** --microbiology --MI; \***Clostridium botulinum** --chemistry--CH; \*Neurotoxins--chemistry--CH ; Amino Acid Sequence; Antibodies, Monoclonal--chemistry--CH; Antigens, Bacterial--immunology--IM; Botulinum Toxins--biosynthesis--BI; Clostridium **botulinum** --classification--CL; Clostridium **botulinum** --metabolism--ME; Endopeptidases; **Epitopes** --immunology--IM; Hydrolysis; Infant; Japan; Molecular Sequence Data; Neurotoxins--biosynthesis--BI; Protein Binding; Synaptosomes--chemistry--CH

CAS Registry No.: 0 (Antibodies, Monoclonal); 0 (Antigens, Bacterial) ; 0 (Botulinum Toxins); 0 (Epitopes); 0 (Neurotoxins)

Enzyme No.: EC 3.4.- (Endopeptidases)

Record Date Created: 19960314

Record Date Completed: 19960314

09184830 PMID: 1723713

**The structure and function of botulinum type C neurotoxin]**

Kimura K

Department of Microbiology, Sapporo Medical College, Japan.

Hokkaido igaku zasshi The Hokkaido journal of medical science (JAPAN)

Nov 1991, 66 (6) p841-8, ISSN 0367-6102 Journal Code: 17410290R

Document type: Journal Article ; English Abstract

Languages: JAPANESE

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The structure gene for **botulinum** type C neurotoxin was cloned from the toxigenic bacteriophage obtained from *Clostridium botulinum* type C, and the whole nucleotide sequence was determined. The nucleotide sequence contained a single open reading frame coding for 1,291 amino acids corresponding to a polypeptide with a molecular weight of 149,000. The signal peptide was not found after the first methionine residue. Upstream of the ATG codon, sequences predicted as a Shine-Dalgarno and a promoter were found. When the deduced amino acid sequence of type C toxin was compared with those of type A and D **botulinum** toxins and tetanus toxin, type C toxin shared about 52% identity with type D toxin, but shared only about 33% identity with type A and tetanus toxins. The structure and function of type C toxin were estimated from the results of **epitope** map with monoclonal antibodies and DNA thermal stability map.

Descriptors: **Botulinum** Toxins; Amino Acid Sequence; Base Sequence; **Botulinum** Toxins--chemistry--CH; **Botulinum** Toxins--genetics--GE; Codon ; DNA, Bacterial; **Epitopes** ; Molecular Sequence Data; Molecular Weight

Molecular Sequence Databank No.: GENBANK/D90210

CAS Registry No.: 0 (Botulinum Toxins); 0 (Codon); 0 (DNA, Bacterial); 0 (Epitopes)

03810050 PMID: 4134872

**Immunological heterogeneity of Clostridium botulinum type B toxins.**

Shimizu T; Kondo H; Sakaguchi G

Japanese journal of medical science & biology (JAPAN) Apr 1974, 27  
(2) p99-100, ISSN 0021-5112 Journal Code: 0243706

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Descriptors: **Botulinum** Toxins; Animals; **Botulinum** Antitoxin;  
Clostridium **botulinum** --immunology--IM; **Epitopes** ; Guinea Pigs  
--immunology--IM; Horses--immunology--IM; Immunization; Neutralization  
Tests

CAS Registry No.: 0 (Botulinum Antitoxin); 0 (Botulinum Toxins); 0  
(Epitopes)

Record Date Created: 19740907

Record Date Completed: 19740907

08349031 PMID: 2479960

**Structure and function of botulinum toxin]**

Shuto B; Kubo S

Tanpakushitsu kakusan koso. Protein, nucleic acid, enzyme (JAPAN) Sep  
1989, 34 (11) p1342-50, ISSN 0039-9450 Journal Code: 0413762

Document type: Journal Article; Review; Review, Tutorial

Languages: JAPANESE

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

(47 Refs.)

Descriptors: **Botulinum** Toxins; Acetylcholine--metabolism--ME; Adenosine  
Diphosphate--metabolism--ME; Amino Acid Sequence; Animals; **Botulinum**  
Toxins--immunology--IM; **Botulinum** Toxins--toxicity--TO; Depression,  
Chemical; **Epitopes** ; Molecular Sequence Data; Molecular Structure; Neurons  
--drug effects--DE; Neurotransmitters--metabolism--ME

CAS Registry No.: 0 (Botulinum Toxins); 0 (Epitopes); 0  
(Neurotransmitters); 51-84-3 (Acetylcholine); 58-64-0 (Adenosine  
Diphosphate)

Record Date Created: 19900109

Record Date Completed: 19900109

**Antagonism of the intracellular action of botulinum neurotoxin type A with monoclonal antibodies that map to light-chain epitopes .**

Cenci Di Bello I; Poulain B; Shone C C; Tauc L; Dolly J O

Department of Biochemistry, Imperial College of Science, Technology & Medicine, London, England.

European journal of biochemistry / FEBS (GERMANY) Jan 15 1994, 219 (1-2) p161-9, ISSN 0014-2956 Journal Code: 0107600

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

mAbs were produced in mice against highly purified, renatured light chain (LC) of **botulinum** neurotoxin A (BoNT A) that was immobilised on nitrocellulose to avoid the undesirable use of toxoids. Subcutaneous implants of relatively high amounts (up to 10 micrograms each) of LC allowed its slow release into the systemic circulation and, thus, yielded much higher antibody titres against the underivatized antigen than had hitherto been obtained by conventional immunization. Seven stable hybridoma cell lines were established which secrete mAb of IgG1 and IgG2b subclasses reactive specifically with BoNT A and LC, in native and denatured states, without showing any cross-reactivity with types B, E, F or tetanus toxin. The pronounced reactivities of three mAbs towards refolded LC or intact toxin, observed in immunobinding and precipitation assays, relative to that seen in Western blots imply a preference for conformational **epitopes** . Though mAbs 4, 5 and 7 failed to neutralize the lethality of BoNT in vivo, administration intraneurally of mAb7 prevented the inhibition of transmitter release normally induced by subsequent extracellular administration of BoNT A. Notably, the latter mAb reacted with a synthetic peptide corresponding to amino acids 28-53 in the N-terminus of the LC, a highly conserved region in Clostridial neurotoxins reported to be essential for maintaining the tertiary structure of the chain. Most importantly, when mAbs 4 or 7 were microinjected inside ganglionic neurons of Aplysia, each reversed, though transiently, the blockade of acetylcholine release by the toxin; this novel finding is discussed in relation to the nature of the zinc-dependent protease activity of the toxin.

Tags: In Vitro; Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.

Descriptors: Antibodies, Monoclonal--pharmacology--PD; \* **Botulinum** Toxins--antagonists and inhibitors--AI; \* **Botulinum** Toxins--immunology--IM; \*Neurons--drug effects--DE; \*Neurotoxins--antagonists and inhibitors--AI; Amino Acid Sequence; Animals; Antibodies, Monoclonal--metabolism--ME; Aplysia; Enzyme-Linked Immunosorbent Assay; **Epitopes** --metabolism--ME; Mice; Mice, Inbred BALB C--immunology--IM; Multiple Myeloma; Neurons--physiology--PH; Neurotoxins--immunology--IM; Peptides--chemical synthesis--CS; Peptides--immunology--IM; Tumor Cells, Cultured

CAS Registry No.: 0 (Antibodies, Monoclonal); 0 (Botulinum Toxins); 0 (Epitopes); 0 (Neurotoxins); 0 (Peptides)

Record Date Created: 19940317

Record Date Completed: 19940317

12624415 PMID: 7744367

**Structure and function of botulinum toxin]**

Fujii N

Department of Microbiology, Sapporo Medical University School of Medicine, Japan.

Hokkaido igaku zasshi The Hokkaido journal of medical science (JAPAN)

Jan 1995, 70 (1) p19-28, ISSN 0367-6102 Journal Code: 17410290R

Document type: Journal Article; Review; Review, Tutorial ; English  
Abstract

Languages: JAPANESE

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

**Botulinum** toxins (types A to G) inhibit the release of acetylcholine at the neuromuscular junction. These toxins are produced as progenitor toxins of large molecular sizes of 12S (M toxin), 16S (L toxin) and 19S (LL toxin) in culture supernatants. Three different molecular forms have been demonstrated in **botulinum** type A toxin. L and M toxins are recognized in **botulinum** type C and D toxins. Type E toxin is exclusively composed of M toxin. In an alkaline condition, M and L toxins dissociate into neurotoxin and nontoxic components. Nontoxic components consist of nontoxic-nonhemagglutinin component (nontoxic-nonHA) and hemagglutinin (HA). M toxin is made up by association of neurotoxin with nontoxic-nonHA, and L toxin is formed by conjugation of M toxin with HA. HA also consists of several subcomponents. These genes with related functions (progenitor toxin) are closely grouped as operon on the chromosome. Nontoxic-nonHA gene is located only 17 bp (type C) or 27 bp (type E) upstream of the neurotoxin gene. Both genes may be transcribed (right-ward transcription) by a polycistronic mRNA species initiated from a promoter located in the 5'-untranslated **region** of the nontoxic-nonHA gene. The construction of HA subcomponent genes (HA-33, HA-17, HA-25 and HA-53) also appears operon structure. The gene cluster related HA is located 262 bp upstream of nontoxic-nonHA gene of type C and transcribed (left-ward transcription) by the same mRNA from the 5'-noncoding **region** of HA-33 gene. **Botulinum** neurotoxin undergoes cleavage to form a dichain molecule linked through a disulphide bond. The **heavy** chain correlates with the binding of toxin to peripheral synapses, and the **light** chain is associated with the intracellular activity of blocking of acetylcholine release. Fifty amino acids in C-terminal **region** of type C toxin is essential for the binding activity of toxin to the target cells. However, the binding efficiency of type C toxin is not antagonized by the other type of **botulinum** toxins because of low homology of this binding **domain** of type C toxin to other types. Furthermore, five highly homologous **regions** are found in **light** chain among seven neurotoxins. One of these homologous **regions**, sequence HEL-H--, shows strong similarity with the active site of zinc-proteases. The inhibition of acetylcholine release is associated with this protease activity which selectively cleaves the synaptic vesicle membrane proteins. These target membrane proteins are key components of the synaptic vesicle docking and fusion. (ABSTRACT TRUNCATED AT 400 WORDS) (30 Refs.)

Tags: Human

Descriptors: **Botulinum** Toxins--chemistry--CH; Acetylcholine--metabolism--ME; Amino Acid Sequence; Animals; **Botulinum** Toxins--genetics--GE; Molecular Sequence Data; Molecular Weight; Neuromuscular Junction--metabolism--ME

CAS Registry No.: 0 (**Botulinum** Toxins); 51-84-3 (Acetylcholine)

Record Date Created: 19950609

Record Date Completed: 19950609

09696672 PMID: 8486245

**Nucleotide sequence of the gene coding for Clostridium barati type F neurotoxin: comparison with other clostridial neurotoxins.**

Thompson D E; Hutson R A; East A K; Allaway D; Collins M D; Richardson P T

Department of Microbiology, AFRC Institute of Food Research, Reading Laboratory, UK.

FEMS microbiology letters (NETHERLANDS) Apr 1 1993, 108 (2) p175-82, ISSN 0378-1097 Journal Code: 7705721

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The neurotoxin gene from Clostridium barati ATCC43756 was cloned as a series of overlapping polymerase chain reaction (PCR) generated **fragments** using primers designed to conserve toxin sequences previously published. The toxin gene has an open reading frame (ORF) of 1268 amino acids giving a calculated molecular mass of 141,049 Da. The sequence identity between the C. barati ATCC43756 and non-proteolytic C. **botulinum** 202F neurotoxins is 64.2% for the **light** chain and 73.6% for the **heavy** chain. This is much lower than reported identities for the type E neurotoxins from C. **botulinum** and C. butyricum (96% identity between **light** chains and 98.8% between the **heavy** chains). Previously identified conserved **regions** in other **botulin** neurotoxins were also conserved in that of C. barati. An ORF upstream of the toxin coding **region** was revealed. This shows strong homology to the 3' end of the gene coding for the nontoxic-nonhemagglutinin (NTNH) component of the progenitor toxin from C. **botulinum** type C neurotoxin.

Tags: Comparative Study

Descriptors: \*Bacterial Toxins--genetics--GE; \*Clostridium--genetics--GE; \*Genes, Bacterial--genetics--GE; \*Neurotoxins--genetics--GE; \*Tetanus Toxin--genetics--GE; Amino Acid Sequence; Base Sequence; Cloning, Molecular; Conserved Sequence; Molecular Sequence Data; Polymerase Chain Reaction; Sequence Homology, Amino Acid; Tetanus Toxin--classification--CL; Variation (Genetics)

Molecular Sequence Databank No.: GENBANK/X68262

CAS Registry No.: 0 (Bacterial Toxins); 0 (Clostridium barati F toxin); 0 (Neurotoxins); 0 (Tetanus Toxin)

Record Date Created: 19930610

Record Date Completed: 19930610

09579419 PMID: 1485344

**A zinc-protease specific domain in botulinum and tetanus neurotoxins.**

Fujii N; Kimura K; Yokosawa N; Tsuzuki K; Oguma K

Department of Microbiology, Sapporo Medical College, Hokkaido, Japan.

Toxicon - official journal of the International Society on Toxinology (ENGLAND) Nov 1992, 30 (11) p1486-8, ISSN 0041-0101 Journal Code: 1307333

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Neurotoxins produced by *Clostridium botulinum* are classified into groups (A to G) based on their serological nature. They consist of two subunits, **heavy** and **light** chains, linked by one or more disulphide bridges. The **light** chain is responsible for the blocking of acetylcholine release. Amino acid sequences of **light** chains have already been reported for *botulinum* toxins types A, C, D and E. Five highly homologous **regions** are found between these four toxins. One of these homologous **regions**, sequence HELIHSL, shows strong similarity with the active site of zinc-proteases. We suggest that inhibition of acetylcholine release might be associated with this protease activity.

Descriptors: **Botulinum** Toxins--analysis--AN; \*Metalloendopeptidases--analysis--AN; \*Neurotoxins--analysis--AN; \*Tetanus Toxin--analysis--AN; Acetylcholine--metabolism--ME; Amino Acid Sequence; Molecular Sequence Data; Synapses--enzymology--EN

CAS Registry No.: 0 (Botulinum Toxins); 0 (Neurotoxins); 0 (Tetanus Toxin); 51-84-3 (Acetylcholine)

Enzyme No.: EC 3.4.24 (Metalloendopeptidases)

Record Date Created: 19930212

Record Date Completed: 19930212

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09445677 PMID: 1388670

**Pepsin fragmentation of botulinum type E neurotoxin: isolation and characterization of 112, 48, 46, and 16 kD fragments .**

Gimenez J A; DasGupta B R

Department of Food Microbiology and Toxicology, University of Wisconsin, Madison 53706.

Journal of protein chemistry (UNITED STATES) Jun 1992, 11 (3)  
p255-64, ISSN 0277-8033 Journal Code: 8217321

Contract/Grant No.: NS17742; NS; NINDS

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Controlled digestion of approximately 150 kD single chain **botulinum** type E neurotoxin with pepsin at pH 6.0 produced 112, 48, 46, and 16 kD **fragments** . These were chromatographically purified; their locations in the approximately 1300 amino acid residue long neurotoxin were determined by identifying the amino terminal 10 residues of 112 and 48 kD **fragments** , 50 residues of 46 kD **fragment** , and 59 residues of 16 kD **fragment** . The 48 and 112 kD **fragments** contain the N-terminal segment of the neurotoxin (i.e., residue no. 1 to approximately 425 and 1 to approximately 990, respectively), the 46 kD **fragment** corresponds to approximately 407 residues of the C-terminal **region** , and the 16 kD **fragment** contains the approximately 140 residues from a segment nearer to the C-terminus. The 48 kD **fragment** is similar to the approximately 50 kD N-terminal **light** chain of the approximately 150 kD dichain neurotoxin, which is generated by tryptic cleavage of the approximately 150 kD single chain neurotoxin, and is separated from the approximately 100 kD C-terminal **heavy** chain by dithiothreitol (DTT) reduction of an intrachain disulfide bond in the presence of 2 M urea (Sathyamoorthy and DasGupta, J. Biol. Chem. 260, 10461, 1985). The pepsin-generated 48 kD **fragment** , unlike the **light** chain, ~~was isolated without exposure to DTT and urea.~~ The single chain 112 kD **fragment** following trypsin digestion yielded 48 and 60 kD **fragments** that were separable after DTT reduction of the intrachain disulfide which links them. The N-terminal residues of the smaller **fragment** were identical to that of the single chain 150 kD neurotoxin; the single chain 112 kD **fragment** is therefore the neurotoxin minus the approximately 50 kD C-terminal half of the **heavy** chain. The biological activities of the 48 and 112 kD **fragments** can be demonstrated in permeabilized PC12 cells (Lomneth et al., J. Neurochem. 57, 1413, 1991); they inhibit norepinephrine release.

Tags: Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

**Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A.**

Kurazono H; Mochida S; Binz T; Eisel U; Quanz M; Grebenstein O; Wernars K ; Poulain B; Tauc L; Niemann H

Institute for Microbiology, Federal Research Center for Virus Diseases of Animals, Tubingen, Federal Republic of Germany.

Journal of biological chemistry (UNITED STATES) Jul 25 1992, 267 (21)

p14721-9, ISSN 0021-9258 Journal Code: 2985121R

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

To define conserved domains within the light (L) chains of clostridial neurotoxins, we determined the sequence of botulinum neurotoxin type B (BoNT /B) and aligned it with those of tetanus toxin (TeTx) and BoNT /A, BoNT /C1, BoNT /D, and BoNT /E. The L chains of BoNT /B and TeTx share 51.6% identical amino acid residues whereas the degree of identity to other clostridial neurotoxins does not exceed 36.5%. Each of the L chains contains a conserved motif, HExxHxxH, characteristic for metalloproteases. We then generated specific 5'- and 3'-deletion mutants of the L chain genes of TeTx and BoNT /A and tested the biological properties of the gene products by microinjection of the corresponding mRNAs into identified presynaptic cholinergic neurons of the buccal ganglia of Aplysia californica. Toxicity was determined by measurement of neurotransmitter release, as detected by depression of postsynaptic responses to presynaptic stimuli (Mochida, S., Poulain, B., Eisel, U., Binz, T., Kurazono, H., Niemann, H., and Tauc, L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7844-7848). Our studies allow the following conclusions. 1) Residues Cys439 of TeTx and Cys430 of BoNT /A, both of which participate in the interchain disulfide bond, play no role in the toxification reaction. 2) Derivatives of TeTx that lacked either 8-amino- or 65 carboxyl-terminal residues are still toxic, whereas those lacking 10 amino- or 68 carboxyl-terminal residues are nontoxic. 3) For BoNT /A, toxicity could be demonstrated only in the presence of added nontoxic heavy (H) chain. A deletion of 8 amino-terminal or 32 carboxyl-terminal residues from the L chain had no effect on toxicity, whereas a removal of 10 amino-terminal or 57 carboxyl-terminal amino acids abolished toxicity. 4) The synergistic effect mediated by the H chain is linked to the carboxyl-terminal portion of the H chain, as demonstrated by injection of HC-specific mRNA into neurons containing the L chain. This finding suggests that the HC domain of the H chain becomes exposed to the cytosol during or after the putative translocation step of the L chain.

Tags: Support, Non-U.S. Gov't

Descriptors: Botulinum Toxins--toxicity--TO; \*Tetanus Toxin--toxicity--TO; Acetylcholine--metabolism--ME; Amino Acid Sequence; Animals; Aplysia; Base Sequence; Botulinum Toxins--genetics--GE; Depression, Chemical; Genetic Vectors; Microinjections; Molecular Sequence Data; Mutation; RNA, Messenger--metabolism--ME; Sequence Alignment; Tetanus Toxin--genetics--GE; Transcription, Genetic; Translation, Genetic; Xenopus

CAS Registry No.: 0 (Botulinum Toxins); 0 (Genetic Vectors); 0 (RNA, Messenger); 0 (Tetanus Toxin); 51-84-3 (Acetylcholine)

Record Date Created: 19920826

Record Date Completed: 19920826

09067016 PMID: 1930094

**Clostridial neurotoxins: from toxins to therapeutic tools?**

Niemann H; Binz T; Grebenstein O; Kurazono H; Thierer J; Mochida S; Poulain B; Tauc L

Institute for Microbiology, Federal Research Center of Virus Diseases of Animals, Tübingen, Germany.

Behring Institute Mitteilungen (GERMANY) Jul 1991, (89) p153-62,  
ISSN 0301-0457 Journal Code: 0367532

Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Tetanus toxin and **botulinum** toxins are powerful neurotoxins which block neurotransmitter release through an unknown mechanism by means of their **light** chains. The **heavy** chains provide the machinery for neuroselective binding, internalization, retrograde intraaxonal transport, and translocation of the L-chains into the cytosol. We have cloned and sequenced the structural genes of tetanus toxin and of five serologically distinct **botulinum** toxins to identify structurally and functionally conserved subdomains. The minimum essential **domains** of the L-chains of tetanus and **botulinum** toxin type A were identified by combined in vitro transcription and microinjection of L-chain specific mRNA into identified presynaptic neurons of *Aplysia californica*. In addition, a nontoxic mutant of tetanus was generated by replacing histidine(237) by a proline residue. The development of nontoxic neuroselective transporter molecules carrying various marker enzymes is discussed. (36 Refs.)

Tags: Comparative Study; Human; Support, Non-U.S. Gov't

Descriptors: **Botulinum** Toxins--genetics--GE; \**Clostridium*; \*Neurotoxins--therapeutic use--TU; \*Tetanus Toxin--genetics--GE; Amino Acid Sequence; Animals; **Botulinum** Toxins--therapeutic use--TU; *Clostridium*--genetics--GE; Genes, Structural, Bacterial; Molecular Sequence Data; RNA, Messenger--genetics--GE; Sequence Homology, Nucleic Acid; Synapses--drug effects--DE; Tetanus Toxin--therapeutic use--TU

CAS Registry No.: 0 (*Botulinum* Toxins); 0 (Neurotoxins); 0 (RNA, Messenger); 0 (Tetanus Toxin)

Record Date Created: 19911121

Record Date Completed: 19911121

**Clues to the multi-phasic inhibitory action of botulinum neurotoxins on release of transmitters.**

Dolly J O; Ashton A C; McInnes C; Wadsworth J D; Poulain B; Tauc L; Shone C C; Melling J

Department of Biochemistry, Imperial College, London, U.K.

Journal de physiologie (FRANCE) 1990, 84 (3) p237-46, ISSN 0021-7948 Journal Code: 9309350

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

1. With the aim of gaining insight into the mechanism of  $\text{Ca}^{2+}$ -dependent secretion, inhibition of transmitter release by **botulinum** neurotoxins or their **fragments** was studied at mammalian motor nerve terminals, cerebrocortical synaptosomes and PC-12 cells. 2. Relative to **BoNT** type A, the feeble neuromuscular paralytic activity of its two chains and the lack of activity observed with a proteolytic **fragment**, H2L (lacking H1, the C-terminal half of the **heavy** chain) highlight a requirement of the intact, disulphide-linked dichain protein for efficient targetting (binding/uptake) to peripheral cholinergic nerve endings. 3. In PC-12 cells, the renatured **light** chain alone proved equally potent as the whole toxin in reducing  $\text{Ca}^{2+}$ -evoked noradrenaline release, when digitonin-permeabilization was used to overcome the uptake barrier. Treatment of **BoNT** A with 10 mM dithiothreitol, under non-denaturing conditions, was not very effective in reducing its inter-chain disulphide bond(s) and had little influence on the level of inhibition seen. 4. Altering the intra-synaptosomal concentrations of cyclic nucleotides (c-AMP, c-GMP) or protein kinase C activity failed to affect the reduction of  $\text{Ca}^{2+}$ -dependent  $\text{K}^{+}$ -stimulated noradrenaline release caused by **BoNT** A or B. On the other hand, raising the cytosolic  $\text{Ca}^{2+}$  concentration with the ionophore A23187 reversed the inhibitory effect of **BoNT** A to a greater extent than that of type B, revealing differences in their actions. 5. Whereas **BoNT** -induced decrease of  $\text{Ca}^{2+}$ -dependent  $\text{K}^{+}$ -evoked release of noradrenaline was unaffected by destruction of the actin-based cytoskeleton in synaptosomes with cytochalasin D, disassembly of microtubules with colchicine, nocodazole or griseofulvin antagonised the intracellular action of type B but not A. It is speculated that **BoNT** B blocks transmitter release by interfering with the proposed detachment of synaptic vesicles from microtubules. Establishing the precise involvement of tubulin in the toxin's action may provide a valuable clue to the mechanism of neurotransmitter release or its control.

Tags: Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.

Descriptors: **Botulinum** Toxins--pharmacology--PD; \*Mammals--physiology--PH; \*Neurotoxins--pharmacology--PD; \*Neurotransmitter Uptake Inhibitors--pharmacology--PD; Animals; **Botulinum** Toxins--isolation and purification--IP; Neuromuscular Junction--drug effects--DE; Neuromuscular Junction--physiology--PH; Neurotoxins--isolation and purification--IP; Norepinephrine--metabolism--ME; Synaptic Transmission

08760815 PMID: 1987060

**Immunological characterization of Clostridium butyricum neurotoxin and its trypsin-induced fragment by use of monoclonal antibodies against Clostridium botulinum type E neurotoxin.**

Kozaki S; Onimaru J; Kamata Y; Sakaguchi G

Department of Veterinary Science, College of Agriculture, University of Osaka Prefecture, Japan.

Infection and immunity (UNITED STATES) Jan 1991, 59 (1) p457-9,

ISSN 0019-9567 Journal Code: 0246127

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

We examined the reactivities of Clostridium butyricum neurotoxin to nine monoclonal antibodies against Clostridium **botulinum** type E neurotoxin which recognize the **light** chain or the amino-terminal half (H-1 **fragment**) or the carboxyl-terminal half (H-2 **fragment**) of the **heavy** chain of **botulinum** neurotoxin. Butyricum neurotoxin and its derived chains did not react to two of four monoclonal antibodies recognizing the **light** chain, one of three recognizing the H-1 **fragment**, and one of two recognizing the H-2 **fragment**. The results indicate that the immunological difference between the two neurotoxins is not attributable to a particular portion of the toxin molecule. The **fragment** of butyricum neurotoxin obtained by prolonged tryptic treatment was found to comprise the **light** chain and H-1 **fragment** linked together by a disulfide bond.

Descriptors: Antibodies, Monoclonal--diagnostic use--DU; \* **Botulinum** Toxins--immunology--IM; \*Clostridium--metabolism--ME; \*Neurotoxins--immunology--IM; \* **Peptide Fragments** --immunology--IM; Animals; Antibodies, Monoclonal--immunology--IM; Clostridium--immunology--IM; Mice; Molecular Weight; Trypsin--pharmacology--PD

CAS Registry No.: 0 (Antibodies, Monoclonal); 0 (Botulinum Toxins); 0 (Neurotoxins); 0 (Peptide Fragments)

Enzyme No.: EC 3.4.21.4 (Trypsin)

Record Date Created: 19910220

Record Date Completed: 19910220

08373527 PMID: 2574722

**Multiple domains of botulinum neurotoxin contribute to its inhibition of transmitter release in Aplysia neurons.**

Poulain B; Wadsworth J D; Shone C C; Mochida S; Lande S; Melling J; Dolly J O; Tauc L

Laboratoire de Neurobiologie Cellulaire et Moleculaire, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France.

Journal of biological chemistry (UNITED STATES) Dec 25 1989, 264 (36) p21928-33, ISSN 0021-9258 Journal Code: 2985121R

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The binding, internalization, and inhibition of transmitter release by botulinum neurotoxin ( BoNT ) was investigated using the intact toxin, its heavy (HC) or light (LC) chains, and a proteolytic fragment thereof. In Aplysia neurons, blockade of acetylcholine release upon external application of BoNT types A or E was prevented by reducing the temperature to 10 degrees C, due to arresting intoxication at the membrane binding step. At this low temperature, type A HC, H2 (comprised of the N-terminal of HC), or H2L (H2 disulfide-linked to LC) antagonized the neuromuscular action of BoNT A or E, indicating that the latter bind saturably to common ecto-acceptor via the H2 region . In contrast, H2L was unable to counteract BoNT -induced paralysis at the murine neuromuscular junction. In accordance with this species difference, unlike native BoNT , saturable binding of 125I-labeled H2L could not be detected in mammalian peripheral or central nerve terminals. Possibly, more stringent structural requirements form the basis of the toxin's greater effectiveness in inhibiting neurotransmission at mouse nerve muscle synapses than Aplysia nerve terminals. In further identification of functional domains in the toxin, an unprocessed single-chain form of BoNT type E was found to be ineffective when applied extra- or intracellularly to Aplysia neurons. Notably, bath application of the latter to a neuron preinjected with HC, but not H2L or LC, resulted in a blockade of release. This shows that the single-chain species can become internalized and requires, not only LC, but also processed HC for its inhibitory action; consistently, the proteolyzed form of BoNT E was active.

Tags: In Vitro; Support, Non-U.S. Gov't; Support, U.S. Gov't, Non-P.H.S.

Descriptors: Acetylcholine--secretion--SE; \* Botulinum Toxins --pharmacology--PD; \*Neuromuscular Junction--physiology--PH; \*Neurons --secretion--SE; \*Neurotoxins; \*Neurotransmitters--secretion--SE; Acetylcholinesterase--metabolism--ME; Animals; Aplysia; Botulinum Toxins --metabolism--ME; Diaphragm--innervation--IR; Macromolecular Systems; Mice; Motor Endplate--drug effects--DE; Motor Endplate--physiology--PH; Motor Neurons--metabolism--ME; Neuromuscular Junction--drug effects--DE; Neurons --drug effects--DE; Peptide Fragments --pharmacology--PD; Phrenic Nerve --drug effects--DE; Phrenic Nerve--physiology--PH; Synaptic Transmission --drug effects--DE; Temperature

CAS Registry No.: 0 (Botulinum Toxins); 0 (Macromolecular Systems); 0 (Neurotoxins); 0 (Neurotransmitters); 0 (Peptide Fragments); 51-84-3 (Acetylcholine)

Enzyme No.: EC 3.1.1.7 (Acetylcholinesterase)

Record Date Created: 19900201

Record Date Completed: 19900201

**Inhibition of transmitter release by botulinum neurotoxin A. Contribution of various fragments to the intoxication process.**

Poulain B; Wadsworth J D; Maisey E A; Shone C C; Melling J; Tauc L; Dolly J O

Laboratoire de Neurobiologie Cellulaire et Moleculaire, Centre National de la Recherche Scientifique, Gif-sur-Yvette.

European journal of biochemistry / FEBS (GERMANY, WEST) Oct 20 1989, 185 (1) p197-203, ISSN 0014-2956 Journal Code: 0107600

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

1. The contribution of a proteolytic fragment (H2L) of botulinum neurotoxin type A (comprised of the aminoterminal region of the heavy-chain disulphide-linked to the light chain) to inhibition of neurotransmitter release was investigated, using central cholinergic synapses of Aplysia, rodent nerve-diaphragm preparations and cerebrocortical synaptosomes. 2. No reduction in neurotransmitter release was observed following external application to these preparations of highly purified H2L or after intracellular injection into Aplysia neurons. 3. The lack of activity was not the result of alteration in the light chain of H2L during preparation of the latter because (a) renaturation of this light chain with intact heavy chain produced a toxic di-chain form and (b) simultaneous application of heavy chain and light chain from H2L inhibited transmitter release in Aplysia. 4. Bath application of H2L and heavy chain together inhibited release of transmitter; however, at the neuromuscular junction the potency of this mixture was much lower than that of native toxin. A similar blockade resulted when heavy chain was applied intracellularly and H2L added to the bath, demonstrating that H2L is taken up into cholinergic neurons of Aplysia. This uptake is shown to be mediated by the amino-terminal moiety of heavy chain (H2), because bath application of light chain plus H2 led to a decrease in acetylcholine release from a neuron that had been injected with heavy chain. 5. A role within the neuron is implicated for a carboxy-terminal portion of heavy chain (H1) since intracellular injection of light chain and H2 did not affect transmitter release. Although the situation is unclear in mammalian nerves, these collective findings indicate that blockade of transmitter release in Aplysia neurons requires the intracellular presence of light chain and H1 (by inference), whilst H2 contributes to the internalization step.

Tags: Support, Non-U.S. Gov't

Descriptors: Botulinum Toxins--toxicity--TO; \*Neurotoxins--toxicity--TO; \*Neurotransmitters--secretion--SE; \*Peptide Fragments --toxicity--TO; Acetylcholine--secretion--SE; Animals; Aplysia; Synaptic Transmission

CAS Registry No.: 0 (Botulinum Toxins); 0 (Neurotoxins); 0 (Neurotransmitters); 0 (Peptide Fragments); 51-84-3 (Acetylcholine)

Record Date Created: 19891204

Record Date Completed: 19891204

Botulinum neurotoxin type A radiolabeled at either the light or the heavy chain.

Dekleva M L; DasGupta B R; Sathyamoorthy V

Food Research Institute, University of Wisconsin, Madison 53706.

Archives of biochemistry and biophysics (UNITED STATES) Oct 1989, 274

(1) p235-40, ISSN 0003-9861 Journal Code: 0372430

Contract/Grant No.: NS 17742; NS; NINDS; NS 24545; NS; NINDS

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

**Botulinum** neurotoxin (NT) has two distinct structural regions called L and H chains (approximately 50 and approximately 100 kDa, respectively). Although the H chain is responsible for binding of the NT to neuronal cells, it is not known which of the subunits is internalized and therefore responsible for causing the blockage of acetylcholine release in susceptible neuronal cells. In this report we describe for the first time the preparation of type A NT which is selectively radiolabeled at either the L or the H chain subunit. Such NT preparations will be useful as tools for determining the distribution of L and H chains in poisoned neuronal cells and the role that each subunit plays in inducing toxicity. The L and H chains of the NT (approximately 150 kDa) were separated, purified, and then individually radiolabeled by reductive methylation of the lysine residues using [3H]- or [14C]formaldehyde. The labeled L and H chains were reconstituted with the complementary unlabeled L and H chains. Formation of -S-S- and noncovalent bonds between the L and H chains regenerated the approximately 150 kDa NT. Autoradiographs of sodium dodecyl sulfate polyacrylamide gels confirmed that each reconstituted NT preparation was labeled at only one subunit chain. NT selectively labeled at either the L or the H chain had specific radioactivities of ca. 25-30 and 45-55 microCi/mumol, respectively, and toxicity (mouse LD50/mg protein) values of  $2.2 \pm 1.1 \times 10(7)$  and  $3.0 \pm 1.0 \times 10(7)$ , respectively. A linear increase in the specific radioactivity of L and H chain subunits was observed with increasing concentrations of 3H- or 14C-labeled formaldehyde in the reaction mixture and with increasing concentrations of L or H chain in the reaction mixture.

Tags: Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

Descriptors: **Botulinum** Toxins; \*Neurotoxins; Carbon Radioisotopes; Electrophoresis, Polyacrylamide Gel; Formaldehyde; Indicators and Reagents; Isotope Labeling--methods--MT; Macromolecular Systems; Tritium

CAS Registry No.: 0 (Botulinum Toxins); 0 (Carbon Radioisotopes); 0 (Indicators and Reagents); 0 (Macromolecular Systems); 0 (Neurotoxins); 10028-17-8 (Tritium); 50-00-0 (Formaldehyde)

Record Date Created: 19891012

Record Date Completed: 19891012

09894576 PMID: 1369426

**Purification of bacterial exotoxins. The case of botulinum, tetanus, anthrax, pertussis and cholera toxins.**

Pasechnik V A; Shone C C; Hambleton P

PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, UK.

Bioseparation (NETHERLANDS) 3 (5) p267-83, ISSN 0923-179X

Journal Code: 9011423

Document type: Journal Article; Review; Review, Tutorial

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: BIOTECHNOLOGY

Bacterial protein toxins and their fragments have been isolated and purified for various reasons, including the development of efficient vaccines and for methods of identification of bacterial agents causing disease. This activity continues today but a new area of bacterial protein toxin research has recently emerged. Since it was shown that toxin molecules comprise several types of biological activity within their structural domains, it was suggested to use these domains (and their combinations) as biochemical tools for developing novel agents for disease imaging and and/or relieving. In this way eukaryotic cell-receptor specific fusion toxins have been developed to prevent malignancy in human. While human clinical trials of these preparations have only recently begun, the preliminary clinical findings are promising. Also fusion proteins which combine independent immunodominant **epitopes** from different antigens have also been developed thus opening a way for the generation of new vaccines for both human and veterinary use. Receptor binding fragments of microbial toxins when combined with other molecules may be useful in delivering these molecules into the cell. In this way novel agents may be developed with a potential for inducing specific changes at the molecular level for the correction of metabolic disorders causing human and animal diseases. Bacterial protein toxins such as anthrax, botulinum, cholera, pertussis and tetanus for which considerable progress has been achieved in structure-function analysis are promising candidates for such research. Particularly exciting appears the idea of extending this research to the cells of the nervous system, exploiting the unique specificity of the botulinum or tetanus toxin fragments which may bring long desired methods for treatment of various disorders of the nervous system. Data on functional domains of these toxins as well as methods of purification of the whole toxins and their fragments are considered in this review as they form a base for their further structure-function analysis and engineering applications. (78 Refs.)

Tags: Human

Descriptors: \*Bacterial Toxins--isolation and purification--IP; \*Exotoxins--isolation and purification--IP; Animals; Bacillus anthracis --metabolism--ME; **Botulinum Toxins** --isolation and purification--IP; Cholera Toxin--isolation and purification--IP; Chromatography, Liquid; Tetanus Toxin--isolation and purification--IP; Virulence Factors, Bordetella--isolation and purification--IP

CAS Registry No.: 0 (Bacterial Toxins); 0 (Botulinum Toxins); 0 (Exotoxins); 0 (Tetanus Toxin); 0 (Virulence Factors, Bordetella); 0 (anthrax toxin); 9012-63-9 (Cholera Toxin)

Record Date Created: 19931230

Record Date Completed: 19931230

09184830 PMID: 1723713

[The structure and function of botulinum type C neurotoxin]

Kimura K

Department of Microbiology, Sapporo Medical College, Japan.

Hokkaido igaku zasshi The Hokkaido journal of medical science (JAPAN)

Nov 1991, 66 (6) p841-8, ISSN 0367-6102 Journal Code: 17410290R

Document type: Journal Article ; English Abstract

Languages: JAPANESE

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The structure gene for botulinum type C neurotoxin was cloned from the toxigenic bacteriophage obtained from Clostridium botulinum type C, and the whole nucleotide sequence was determined. The nucleotide sequence contained a single open reading frame coding for 1,291 amino acids corresponding to a polypeptide with a molecular weight of 149,000. The signal peptide was not found after the first methionine residue. Upstream of the ATG codon, sequences predicted as a Shine-Dalgarno and a promoter were found. When the deduced amino acid sequence of type C toxin was compared with those of type A and D botulinum toxins and tetanus toxin, type C toxin shared about 52% identity with type D toxin, but shared only about 33% identity with type A and tetanus toxins. The structure and function of type C toxin were estimated from the results of **epitope** map with monoclonal antibodies and DNA thermal stability map.

Descriptors: **Botulinum Toxins** ; Amino Acid Sequence; Base Sequence; **Botulinum Toxins** --chemistry--CH; **Botulinum Toxins** --genetics--GE; Codon ; DNA, Bacterial; **Epitopes** ; Molecular Sequence Data; Molecular Weight

Molecular Sequence Databank No.: GENBANK/D90210

CAS Registry No.: 0 (Botulinum Toxins); 0 (Codon); 0 (DNA, Bacterial); 0 (Epitopes)

Record Date Created: 19920317

Record Date Completed: 19920317

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08862180 PMID: 1707941

[Molecular structure and function of Clostridium botulinum neurotoxin]

Kozaki S

Department of Veterinary Science, University of Osaka Prefecture.

Seikagaku. The Journal of Japanese Biochemical Society (JAPAN) Dec 1990

, 62 (12) p1496-500, ISSN 0037-1017 Journal Code: 0413564

Document type: Journal Article

Languages: JAPANESE

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Descriptors: **Botulinum Toxins** --toxicity--TO; Acetylcholine--metabolism  
--ME; Amino Acid Sequence; Animals; **Botulinum Toxins** --metabolism--ME;  
**Epitopes** ; Molecular Sequence Data; Molecular Structure  
CAS Registry No.: 0 (Botulinum Toxins); 0 (Epitopes); 51-84-3  
(Acetylcholine)

Record Date Created: 19910523

Record Date Completed: 19910523

06563585 PMID: 6204594

**Comparison of antigenicity of toxins produced by Clostridium botulinum type C and D strains.**

Ochanda J O; Syuto B; Oguma K; Iida H; Kubo S

Applied and environmental microbiology (UNITED STATES) Jun 1984, 47

(6) p1319-22, ISSN 0099-2240 Journal Code: 7605801

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

C1 neurotoxin of Clostridium botulinum strains C-Stockholm (C-ST), C beta-Yoichi, C-468, CD6F, and C-CB19 and type D toxin of strains D-1873 and D-CB16 were purified by gel filtration, ion exchange, and affinity chromatographies. The purified toxins had di-chain structure made of heavy and light chains. The toxins of C beta-Yoichi, C-468, CD6F, and C-CB19 reacted with anti-C-ST heavy chain and anti-C-ST light chain in immunodiffusion tests and enzyme-linked immunosorbent assay, whereas D-CB16 toxin reacted with anti-D-1873 heavy chain and anti-D-1873 light chain. However, C-6813 toxin reacted with anti-D-1873 heavy chain and anti-C-ST light chain but not with anti-C-ST heavy chain or anti-D-1873 light chain immunoglobulin G. These results indicate common antigens in the heavy chains of C-6813 and D-1873 toxins and in the light chains of C-6813 and C-ST toxins. Further, they provide evidence for heterogeneity within type C1 toxin subunits.

Tags: Comparative Study

Descriptors: Antigens, Bacterial--immunology--IM; \* Botulinum Toxins --immunology--IM; \* Clostridium botulinum --immunology--IM; Animals; Antigens, Bacterial--analysis--AN; Botulinum Toxins --isolation and purification--IP; Chromatography, Affinity; Chromatography, Gel; Chromatography, Ion Exchange; Clostridium botulinum --classification--CL; Electrophoresis, Polyacrylamide Gel; Enzyme-Linked Immunosorbent Assay; Epitopes--analysis--AN; Immunodiffusion; Immunoglobulins, gamma-Chain --analysis--AN; Mice; Neurotoxins--immunology--IM; Neurotoxins--isolation and purification--IP; Serotyping

CAS Registry No.: 0 (Antigens, Bacterial); 0 (Botulinum Toxins); 0 (Epitopes); 0 (Immunoglobulins, gamma-Chain); 0 (Neurotoxins)

Record Date Created: 19840813

Record Date Completed: 19840813



US005919665A



**United States Patent** [19]  
**Williams**

[11] **Patent Number:** **5,919,665**  
 [45] **Date of Patent:** **Jul. 6, 1999**

[54] **VACCINE FOR CLOSTRIDIUM BOTULINUM NEUROTOXIN**

[75] **Inventor:** James A. Williams, Madison, Wis.

[73] **Assignee:** Ophidian Pharmaceuticals, Inc.,  
 Madison, Wis.

[21] **Appl. No.:** 08/405,496

[22] **Filed:** Mar. 16, 1995

#### Related U.S. Application Data

[63] Continuation-in-part of application No. 08/329,154, Oct. 25, 1994, abandoned, which is a continuation-in-part of application No. 08/161,907, Dec. 2, 1993, Pat. No. 5,601,823, which is a continuation-in-part of application No. 08/985,321, Dec. 4, 1992, which is a continuation-in-part of application No. 07/429,791, Oct. 31, 1989, Pat. No. 5,196,193.

[51] **Int. Cl.**<sup>6</sup> ..... C07K 19/00; C12N 1/20;  
 C12P 1/00

[52] **U.S. Cl.** ..... 435/71.1; 435/252.3; 435/320.1;  
 530/825; 530/350; 536/23.4

[58] **Field of Search** ..... 435/252.3, 320.1,  
 435/71.1; 536/23.4; 530/825, 350

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*Attorney, Agent, or Firm*—Medlen & Carroll, LLP

[57]

#### ABSTRACT

The present invention includes recombinant proteins derived from *Clostridium botulinum* toxins. In particular, soluble recombinant *Clostridium botulinum* type A toxin proteins are provided. Methods which allow for the isolation of recombinant proteins free of significant endotoxin contamination are provided. The soluble, endotoxin-free recombinant proteins are used as immunogens for the production of vaccines and antitoxins. These vaccines and antitoxins are useful in the treatment of humans and other animals at risk of intoxication with clostridial toxin.

10 Claims, 29 Drawing Sheets

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-continued

Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn  
 1205 1210 1215

Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr  
 1220 1225 1230

Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly  
 1235 1240 1245

Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser  
 1250 1255 1260

Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys  
 1265 1270 1275 1280

Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu  
 1285 1290 1295

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site  
 (B) LOCATION: 12  
 (D) OTHER INFORMATION: /note= "The asparagine residue at  
 this position contains an amide group."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Cys Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn  
 1 5 10

## (2) INFORMATION FOR SEQ ID NO:30:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

His His His His His  
 1 5

## I claim:

1. A soluble fusion protein comprising a non-toxin protein sequence and a portion of the *Clostridium botulinum* type A toxin, said portion of the *Clostridium botulinum* type A toxin comprising a portion of the sequence of SEQ ID NO:28.
2. The fusion protein of claim 1, wherein said portion of the *Clostridium botulinum* type A toxin sequence comprises SEQ ID NO:23.
3. The fusion protein of claim 1, wherein said non-toxin protein sequence comprises a poly-histidine tract.
4. The fusion protein of claim 3, which comprises SEQ ID NO:26.
5. The fusion protein of claim 1, wherein said fusion protein is substantially endotoxin-free.
6. A host cell containing a recombinant expression vector, said vector encoding a protein comprising at least a portion of a *Clostridium botulinum* type A toxin protein sequence of

SEQ ID NO:28, and wherein said host cell is capable of expressing said protein as a soluble protein in said host cell at a level greater than or equal to 0.75% of the total cellular protein.

7. The host cell of claim 6, wherein said portion of a toxin comprises SEQ ID NO:23.

8. The host cell of claim 6, wherein said fusion protein comprises SEQ ID NO:26.

9. The host cell of claim 6, wherein said host cell is capable of expressing said protein in said host cell at a level greater than or equal to 20% of the total cellular protein.

10. A soluble fusion protein, comprising at least a portion of *Clostridium botulinum* C fragment linked to a poly-histidine tag.

\* \* \* \* \*



US005919665A

**United States Patent** [19]  
**Williams**

[11] **Patent Number:** **5,919,665**  
 [45] **Date of Patent:** **Jul. 6, 1999**

[54] **VACCINE FOR CLOSTRIDIUM BOTULINUM NEUROTOXIN**

[75] **Inventor:** James A. Williams, Madison, Wis.

[73] **Assignee:** Ophidian Pharmaceuticals, Inc., Madison, Wis.

[21] **Appl. No.:** 08/405,496

[22] **Filed:** Mar. 16, 1995

#### Related U.S. Application Data

[63] Continuation-in-part of application No. 08/329,154, Oct. 25, 1994, abandoned, which is a continuation-in-part of application No. 08/161,907, Dec. 2, 1993, Pat. No. 5,601,823, which is a continuation-in-part of application No. 08/985,321, Dec. 4, 1992, which is a continuation-in-part of application No. 07/429,791, Oct. 31, 1989, Pat. No. 5,196,193.

[51] **Int. Cl.**<sup>6</sup> ..... C07K 19/00; C12N 1/20; C12P 1/00

[52] **U.S. Cl.** ..... 435/71.1; 435/252.3; 435/320.1; 530/825; 530/350; 536/23.4

[58] **Field of Search** ..... 435/252.3, 320.1, 435/71.1; 536/23.4; 530/825, 350

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#### [57] ABSTRACT

The present invention includes recombinant proteins derived from *Clostridium botulinum* toxins. In particular, soluble recombinant *Clostridium botulinum* type A toxin proteins are provided. Methods which allow for the isolation of recombinant proteins free of significant endotoxin contamination are provided. The soluble, endotoxin-free recombinant proteins are used as immunogens for the production of vaccines and antitoxins. These vaccines and antitoxins are useful in the treatment of humans and other animals at risk of intoxication with clostridial toxin.

10 Claims, 29 Drawing Sheets

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Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn  
                   1205                  1210                  1215

Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr  
                   1220                  1225                  1230

Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly  
                   1235                  1240                  1245

Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser  
                   1250                  1255                  1260

Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys  
                   1265                  1270                  1275                  1280

Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu  
                   1285                  1290                  1295

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## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 12
- (D) OTHER INFORMATION: /note= "The asparagine residue at this position contains an amide group."

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Cys Gln Thr Ile Asp Gly Lys Lys Tyr Tyr Phe Asn  
   1                  5                  10

## (2) INFORMATION FOR SEQ ID NO:30:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

His His His His His  
   1                  5

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I claim:

1. A soluble fusion protein comprising a non-toxin protein sequence and a portion of the *Clostridium botulinum* type A toxin, said portion of the *Clostridium botulinum* type A toxin comprising a portion of the sequence of SEQ ID NO:28.
2. The fusion protein of claim 1, wherein said portion of the *Clostridium botulinum* type A toxin sequence comprises SEQ ID NO:23.
3. The fusion protein of claim 1, wherein said non-toxin protein sequence comprises a poly-histidine tract.
4. The fusion protein of claim 3, which comprises SEQ ID NO:26.
5. The fusion protein of claim 1, wherein said fusion protein is substantially endotoxin-free.
6. A host cell containing a recombinant expression vector, said vector encoding a protein comprising at least a portion of a *Clostridium botulinum* type A toxin protein sequence of

SEQ ID NO:28, and wherein said host cell is capable of expressing said protein as a soluble protein in said host cell at a level greater than or equal to 0.75% of the total cellular protein.

7. The host cell of claim 6, wherein said portion of a toxin comprises SEQ ID NO:23.

8. The host cell of claim 6, wherein said fusion protein comprises SEQ ID NO:26.

9. The host cell of claim 6, wherein said host cell is capable of expressing said protein in said host cell at a level greater than or equal to 20% of the total cellular protein.

10. A soluble fusion protein, comprising at least a portion of *Clostridium botulinum* C fragment linked to a poly-histidine tag.

\* \* \* \* \*



US005939070A

**United States Patent** [19]  
**Johnson et al.**

[11] **Patent Number:** **5,939,070**  
[45] **Date of Patent:** **Aug. 17, 1999**

[54] **HYBRID BOTULINAL NEUROTOXINS**

[75] **Inventors:** **Eric A. Johnson**, Madison; **Michael C. Goodnough**, Stoughton; **Marite Bradshaw**, Madison, all of Wis.

[73] **Assignee:** **Wisconsin Alumni Research Foundation**, Madison, Wis.

[21] **Appl. No.:** **08/739,477**

[22] **Filed:** **Oct. 28, 1996**

[51] **Int. Cl.<sup>6</sup>** ..... **A61K 39/385**; **A61K 39/08**;  
C12P 21/06; C12N 9/52

[52] **U.S. Cl.** ..... **424/194.1**; **424/239.1**;  
435/220; 435/842; 530/350; 530/402; 530/412;  
530/825; 536/23.2; 536/23.7; 514/12

[58] **Field of Search** ..... 435/69.1, 172.3,  
435/220, 252.3, 320.1, 842; 514/12; 530/350,  
402, 412, 825; 536/23.2, 23.4, 23.7; 424/247.1,  
194.1, 239.1

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*Primary Examiner*—Rebecca E. Prouty

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*Attorney, Agent, or Firm*—Quarles & Brady LLP

[57] **ABSTRACT**

A hybrid botulinal neurotoxin is disclosed. In one embodiment, the neurotoxin comprises a combination of a botulinal neurotoxin heavy chain and light chain, wherein the light chain and heavy chain are not of the same serotype. A method for creating hybrid neurotoxins comprised of different functional domains is also disclosed.

**14 Claims, 1 Drawing Sheet**

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L11: Entry 62 of 101

File: USPT

Aug 17, 1999

DOCUMENT-IDENTIFIER: US 5939070 A  
TITLE: Hybrid botulinal neurotoxins

Abstract Text (1):


A hybrid botulinal neurotoxin is disclosed. In one embodiment, the neurotoxin comprises a combination of a botulinal neurotoxin heavy chain and light chain, wherein the light chain and heavy chain are not of the same serotype. A method for creating hybrid neurotoxins comprised of different functional domains is also disclosed.

CLAIMS:

1. A hybrid botulinal neurotoxin comprising:

(a) a botulinal neurotoxin light chain; and

(b) a botulinal neurotoxin heavy chain,

wherein the light chain and heavy chain are not of the same serotype and wherein the light and heavy chains are linked by a heterobifunctional thiol/amine linker and wherein the specific toxicity of the neurotoxin is at least 10.sup.6 LD.sub.50 /mg protein in vivo. 

2. The neurotoxin of claim 1 wherein the heavy chain or light chain is isolated from a native botulinal neurotoxin molecule.

3. The neurotoxin of claim 1 wherein the heavy chain or light chain is obtained from a recombinant gene construct.

4. The neurotoxin of claim 1 wherein the heavy and light chains are obtained from recombinant gene constructs.

5. A hybrid botulinal neurotoxin comprising light and heavy chains, which comprise botulinal neurotoxin catalytic, channel forming and receptor binding functional domains, wherein at least two functional domains are from botulinal neurotoxins of different serotypes and wherein the light and heavy chains are linked by a heterobifunctional thiol/amine linker and wherein the specific toxicity of the neurotoxin is at least 10.sup.6 LD.sub.50 /mg protein in vivo.

6. The neurotoxin of claim 5 wherein at least one of the functional domains is isolated from a native botulinal neurotoxin molecule.

7. The neurotoxin of claim 5 wherein at least one of the functional domains is isolated from a recombinant gene construct.

8. The neurotoxin of claim 5 wherein the heavy and light chains are obtained from recombinant gene constructs.

11. A method for creating a hybrid neurotoxin comprising the steps of:

(a) isolating botulinal neurotoxin heavy and light chains from native neurotoxin molecules or a recombinant gene construct; and

linking the heavy and light chains into a hybrid neurotoxin with a heterobifunctional thiol/amine linker wherein the heavy and light chains are not of the same serotype and wherein the specific toxicity of the neurotoxin is at least 10.sup.6 LD.sub.50 /mg protein in vivo.

12. The method of claim 11 wherein the heavy and light chains are obtained from recombinant gene constructs.

13. The method of claim 12 wherein the recombinant gene constructs encode combinations of functional domains that do not occur naturally.

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☐ 1. Document ID: US 6787517 B1

L5: Entry 1 of 19

File: USPT

Sep 7, 2004

DOCUMENT-IDENTIFIER: US 6787517 B1

TITLE: Agent and methods for treating pain

Abstract Text (1):

Agents for treating pain, methods for producing the agents and methods for treating pain by administration to a patient of a therapeutically effective amount of the agent are disclosed. The agent may include a clostridial neurotoxin, a fragment or a derivative thereof, attached to a targeting component, wherein the targeting component is selected from a group consisting of compounds which selectively binds at the alpha-2B or alpha-2B/alpha-2C adrenergic receptor subtype(s) as compared to other binding sites, for example, the alpha-2A adrenergic receptor subtype.

Brief Summary Text (2):

The present invention relates to compositions and methods for treating pain. Particularly, the present invention relates to an agent comprising a neurotoxin, methods for making the agent and methods for treating pain using the agent.

Brief Summary Text (11):

Botulinum Toxin

Brief Summary Text (12):

The anaerobic, gram positive bacterium Clostridium botulinum produces a potent polypeptide neurotoxin, botulinum toxin, which causes a neuromuscular illness in humans and animals referred to as botulism. The spores of Clostridium botulinum are found in soil and can grow in improperly sterilized and sealed food containers of home based canneries, which are the cause of many of the cases of botulism. The effects of botulism typically appear 18 to 36 hours after eating the foodstuffs infected with a Clostridium botulinum culture or spores. The botulinum toxin can apparently pass unattenuated through the lining of the gut and attack peripheral motor neurons. Symptoms of botulinum toxin intoxication can progress from difficulty difficulty walking, swallowing, and speaking to paralysis of the respiratory muscles and death.

Brief Summary Text (13):

Botulinum toxin type A is the most lethal natural biological agent known to man and has a very potent LD<sub>50</sub>. A specific dose of a toxin that would be lethal to 50% of the population of a certain species of animal is called an LD<sub>50</sub>. For example, the estimated LD<sub>50</sub> of botulinum toxin type A (available from Allergan, Allergan, Inc., of Irvine, Calif. as a purified neurotoxin complex under the trade name BOTOX.RTM.) in humans is about 150,000 picograms or about 3,000 units. Interestingly, on a molar basis, botulinum toxin type A is about 1.8 billion times more lethal than diphtheria toxin, about 600 million times more lethal than sodium

cyanide, about 3.0 million times more lethal than cobra toxin and about 12 million times more lethal than cholera toxin. Singh, Critical Aspects of Bacterial Protein Toxins, pages 63-84 (chapter 4) of Natural Toxins II, edited by B. R. Singh et al., Plenum Press, New York (1996).

Brief Summary Text (14):

Seven immunologically distinct botulinum neurotoxins have been characterized, these being respectively botulinum neurotoxin serotypes A, B, C.sub.1, D, E, F and G, each each of which is distinguished by neutralization with type-specific antibodies. The different serotypes of botulinum toxin vary in the animal species that they affect and in the severity and duration of the paralysis they evoke. For example, it has been determined that botulinum toxin type A is 500 times more potent, as measured by the rate of paralysis produced in the rat, than is botulinum toxin type B. Botulinum toxin apparently binds with high affinity to cholinergic motor neurons, is is translocated into the neuron and blocks the release of acetylcholine.

Brief Summary Text (15):

Without wishing to limit the invention to any theory or mechanism of operation, it is believed that the molecular mechanism of toxin intoxication appears to be similar and involve at least three steps or stages, regardless of the serotype. Although, a potential molecular mechanism of toxin intoxication of botulinum toxin is discussed here, other toxins, for example, butyricum toxins, tetani toxins or variants thereof may have the same or substantially similar mechanisms. In the first step of the process, the toxin binds to the presynaptic membrane of the target neuron through a specific interaction between the heavy chain, H chain, and a cell surface receptor; the receptor is thought to be different for each type of botulinum toxin and for tetanus toxin. The carboxyl end segment of the H chain, H.sub.C, appears to be important for targeting of the toxin to the cell surface.

Brief Summary Text (17):

The last step of the mechanism of botulinum toxin activity appears to involve reduction of the disulfide bond joining the heavy chain, H chain, and the light chain, L chain. The entire toxic activity of botulinum and tetanus toxins is contained in the L chain of the holotoxin; the L chain is a zinc (Zn++) endopeptidase, which selectively cleaves proteins essential for recognition and docking of neurotransmitter-containing vesicles with the cytoplasmic surface of the plasma membrane, and fusion of the vesicles with the plasma membrane. Tetanus neurotoxin, botulinum toxin /B/D/F, and /G cause degradation of synaptobrevin (also called vesicle-associated membrane protein (VAMP)), a synaptosomal membrane protein. Most of the VAMP present at the cytoplasmic surface of the synaptic vesicle is removed as a result of any one of these cleavage events. Serotype A and E cleave SNAP-25. Serotype C.sub.1 was originally thought to cleave syntaxin, but was found to cleave syntaxin and SNAP-25. Each toxin specifically cleaves a different bond (except tetanus and type B, which cleave the same bond).

Brief Summary Text (18):

Botulinum toxins have been discovered to have relatively prolonged neurotoxic effects and, as such, have been adapted for use in the treatment of pain, particularly chronic pain, for example, Foster et al. in U.S. Pat. No. 5,989,545, the disclosure of which is incorporated in its entirety herein by reference.

Brief Summary Text (24):

Still further in accordance with the present invention, the therapeutic component substantially interferes with the release of neurotransmitters from a cell or its processes. For example, in one embodiment, the therapeutic component comprises a light chain component, which may be able to inhibit the release of neurotransmitters from a cell. The light chain component may be a light chain or a fragment thereof of a Clostridial toxin such as a botulinum toxin type A, B, C.sub.1, D, E, F, G, a butyricum toxin, a tetani toxin or variants thereof. In another embodiment, the therapeutic component may be a neurotoxin, for example

saporin, through inactivating cellular ribosome functions.

Brief Summary Text (26):

wherein X' is selected from the group consisting of R.sub.4 --C.dbd.C--R.sub.5 and R.sub.4 --C. A six membered carbon ring structure is formed when X' is R.sub.4 --C.dbd.C--R.sub.5. A five membered carbon ring is formed when X' is R.sub.4 --C. R.sub.1, R.sub.2, R.sub.3, R.sub.4 and R.sub.5 are each independently selected from the group consisting of F, Cl, Br, I, OR.sub.6 and H, wherein R.sub.6 is H or an alkyl, including a methyl, an ethyl or a propyl. In one embodiment, the amino acid component may be antibodies raised from an antigen component. The antigen component may include a second extracellular loop of an alpha-2B receptor, which may additionally be conjugated to a keyhole limpet hemocyanin. In one embodiment, the second extracellular loop comprises a peptide fragment comprising an amino acid sequence of KGDQGPQPRGRPQCKLNQE (SEQ ID#1). In another embodiment, the amino acid component may comprise a peptide, polypeptide, protein, protein complex or antibody, which is a variant of a wild type. For example, an amino acid component may be a mutated H chain of botulinum toxin type A which selectively binds to an alpha 2B receptor, as opposed to the wild type which has a higher affinity to motor neuron cell surface proteins. See Goeddel et al. U.S. Pat. No. 5,223,408, the disclosure of which is incorporated in its entirety herein by reference.

Brief Summary Text (27):

Still further in accordance with the present invention, the translocation component is able to facilitate the transfer of a therapeutic component, such as a light chain of a botulinum toxin type A, into the cytoplasm of the target cell. In one embodiment, the translocation component comprises a heavy chain component. The heavy chain component may include a heavy chain or a fragment thereof of a Clostridial toxin such as a botulinum toxin type A, B, C.sub.1, D, E, F, G, a butyricum toxin, a tetani toxin or variants thereof. The fragment of the heavy chain may include an amino end fragment of the heavy chain. In another embodiment, the heavy chain component may comprise at least a fragment of two different neurotoxins. For example, the heavy chain component may comprise an amino end fragment of heavy chain of a botulinum toxin type A, and a carboxyl end fragment of a heavy chain of botulinum toxin type B.

Brief Summary Text (30):

Still further in accordance with the invention, there is provided a method for treating pain comprising the step of administering to a mammal, preferably a human, a therapeutically effective amount of an agent of the present invention. In one embodiment, the therapeutic component and the translocation component of the agent is found together in a botulinum toxin, for example botulinum toxin type A. An agent of the present invention may be administered intrathecally or intramuscularly or subcutaneously, for example at or near the location of pain.

Detailed Description Text (4):

Light chain component comprises a light chain and/or a fragment thereof of a clostridial neurotoxin. The light chain has a molecular weight of about 50 kDa, and may be referred to as L chain or L. A light chain or a fragment thereof may have proteolytic activity.

Detailed Description Text (5):

Heavy chain component comprises a heavy chain and/or a fragment thereof of a clostridial neurotoxin. The full kDa and can be referred to as H chain or as H. The fragment of the heavy chain may be referred to as H.sub.C or H.sub.N.

Detailed Description Text (6):

H.sub.C means a fragment derived from the H chain of a clostridial neurotoxin which is approximately equivalent, for example functionally equivalent, to the carboxyl end fragment of the H chain, or the portion corresponding to that fragment in the intact H chain involved in binding to cell surfaces.

Detailed Description Text (7):

H.sub.N means a fragment derived from the H chain of a clostridial neurotoxin which is approximately equivalent, for example functionally equivalent, to the amino end segment of the H chain, or the portion corresponding to that fragment in the intact in the H chain involved in the translocation of at least the L chain across an intracellular endosomal membrane into a cytoplasm of a cell.

Detailed Description Text (8):

LH.sub.N means a fragment derived from a clostridial neurotoxin that contains the L chain, or a functional fragment thereof coupled to the H.sub.N domain. It can be obtained from the intact clostridial neurotoxin by proteolysis, so as to remove or to modify the H.sub.C domain.

Detailed Description Text (10):

In one embodiment, the therapeutic component substantially interferes with the release of neurotransmitters, preferably neurotransmitters which are involved in pain-signal transmissions, from a neural cell. In a preferred embodiment, the therapeutic component comprises a light chain component. The light chain component may include a light chain of a botulinum toxin, a butyricum toxin, a tetani toxin or or biologically active variants of these toxins. The light chain component may also include a fragment of the mentioned light chains, providing that the fragments are biologically active in a physiological environment. That is, these fragments can substantially interfere with the release of neurotransmitters from a cell or its processes. In a preferred embodiment, the light chain component includes a light chain of a botulinum toxin type A, B, C.sub.1, D, E, F, G or biologically active variants of these serotypes. In another preferred embodiment, the light chain component may even be fragments of the botulinum toxin type A, B, C.sub.1, D, E, E, F, G or the biologically active variants of these serotypes, provided that the fragments themselves are biologically active, for example the fragment is able to interfere with the release of neurotoxins from a cell. As used herein, a variant polypeptide, for example a variant polypeptide, may also mean a modified polypeptide, for example modified light chain.

Detailed Description Text (14):

In a preferred embodiment, the therapeutic component can exert its effect from inside a cell, for example from the cytoplasm. For example, the L chain component, a therapeutic component, exerts its therapeutic effect from inside a neuron. In such a case, it is preferred that the agent further comprises a translocation component. The translocation component is able to facilitate the transfer of at least a part of the agent into the cytoplasm of the target cell. In a preferred embodiment, the translocation component comprises a heavy chain component. The heavy chain component includes a heavy chain or a fragment thereof of a botulinum toxin, a butyricum toxin, a tetani toxin or variants thereof. Preferably, the heavy chain component includes a heavy chain or a fragment thereof of a botulinum toxin type A, B, C.sub.1, D, E, F, G or variants thereof. More preferably, the heavy chain component comprises a fragment of a heavy chain of a botulinum toxin type A. Even more preferably, the fragment is the amino end (or terminal) fragment of heavy chain of botulinum toxin type A which is capable of facilitating the translocation of at least part of the agent, for example the therapeutic component, from inside a vesicle into the cytoplasm of a cell.

Detailed Description Text (15):

In a preferred embodiment, an agent according to this invention comprises a therapeutic component comprising a light chain of a botulinum toxin type A and the translocation component comprising a heavy chain, preferably a fragment thereof, of a botulinum toxin type A, wherein the heavy chain (or the fragment thereof) can assist in the translocation of at least the therapeutic component into a cytoplasm of a cell. In another preferred embodiment, an agent according to this invention comprises a therapeutic component comprising a light chain of a tetani toxin and

the translocation component comprising a heavy chain, preferably a fragment thereof, thereof, of a tetani toxin, wherein the fragment of a heavy chain (or the fragment thereof) can assist in the translocation of at least the therapeutic component into a cytoplasm of a cell. In yet another embodiment, an agent according to this invention comprises a therapeutic component comprising a light chain of one type of botulinum toxin and a translocation component comprising a heavy chain, preferably a fragment of the heavy chain such as the H.sub.N, of another botulinum toxin, constituting a chimeric protein. For example, in one preferred embodiment, an agent in accordance with the invention comprises LH.sub.N whereof the L chain is derived from botulinum toxin type B and the amine end segment of the H chain fragment is derived from botulinum toxin type A. The H.sub.N fragment of the botulinum toxin type A is produced according to the method described by Shone C. C., Hambleton, P., and Melling, J. (1987, Eur. J. Biochem. 167, 175-180) and the L chain of botulinum toxin type B according to the method of Sathyamoorthy, V. and DasGupta, B. R. (1985, J. Biol. Chem. 260, 10461-10466). The free cysteine on the amine end segment of the H chain fragment of botulinum toxin type A is then derivatized by the addition of a ten-fold molar excess of dipyridyl disulphide followed by incubation at 4 degree C. overnight. The excess dipyridyl disulphide and the thiopyridone by product are then removed by desalting the protein over a PD10 column (Pharmacia) into PBS. The derivatized H.sub.N is then concentrated to a protein concentration in excess of 1 mg/ml before being mixed with an equimolar portion of L chain from botulinum toxin type B (>1 mg/ml in PBS). After overnight incubation at room temperature the mixture is separated by size exclusion chromatography over Superose 6 (Pharmacia), and the fractions analyzed by SDS-PAGE. The chimeric LH.sub.N is then available for dramatization to produce a targeted conjugate.

Detailed Description Text (66):

In another embodiment, the amino acid component comprises a variant peptide, polypeptide, protein, protein complex, antibody or a portion thereof of a corresponding wild type. For example, a naturally existing heavy chain of a botulinum toxin is a wild type polypeptide. Preferably, the targeting component comprising a variant of a wild type is able to selectively bind to alpha-2B and/or C receptor and is free of at least one undesired binding property of the wild type sequence of amino acid. For example, in one embodiment, the targeting component comprising a variant of a wild type selectively binds to alpha-2B and/or C receptor and does not bind to a motor neuron cell surface.

Detailed Description Text (70):

In one embodiment, the components of the agents are joined by a spacer component. Spacer components have many functions within this invention. For example, one of the functions of the spacer regions is to provide for adequate distance between the various components so that the components can independently and freely move about, without substantial internal steric hindrance. Such a spacer may comprise, for example, a portion of the botulinum toxin H.sub.C sequence (preferably the portion does not retain the ability to bind to motor neurons or sensory afferent neurons), another sequence of amino acids, or a hydrocarbon moiety. The spacer component may also comprise a proline, serine, threonine and/or cysteine-rich amino acid sequence similar or identical to a human immunoglobulin hinge region. In a preferred embodiment, the spacer region comprises the amino acid sequence of an immunoglobulin .DELTA..sub.1 hinge region; such a sequence has the sequence (from N terminus to C terminus):EPKSCDKTHTCPPCP (SEQ ID#2). In one embodiment, the therapeutic component attaches to the translocation component through a spacer component, and the translocation component also attaches to the targeting component through a spacer component. In a preferred embodiment, the therapeutic component attaches to the translocation component through a spacer component, and the therapeutic component also attaches to the targeting component through a spacer component, or alternatively a disulfide bond. In a more preferred embodiment, the therapeutic component is a light chain of a botulinum toxin type A, the translocation component is a heavy chain, or a fragment thereof, of a botulinum toxin type A which can facilitate the translocation of at least the light chain

into a cytoplasm of a cell, and the targeting component is a molecule which can selectively bind to the alpha-2B and/or C receptors. For example, such a selectively binding molecule may be an agonist or antagonist of the alpha-2B and/or C receptor. An example of such molecule may be represented by the formula: ##STR26##

Detailed Description Text (72):

In one embodiment, the therapeutic component and the translocation component are part of a botulinum toxin, for example botulinum toxin type A. In such a case, a natural, a chemically modified, a recombinant or partially recombinant botulinum toxin type A may be attached to a targeting component, forming the agent of the present invention. Furthermore, it is known in the art that the H.sub.c of the neurotoxin molecule, for example botulinum toxin type A, can be removed from the other segment of the H chain, the H.sub.N, such that the H.sub.N fragment remains disulphide linked to the L chain of the neurotoxin molecule to provide a fragment known as the LH.sub.N. Thus, in one embodiment of the present invention the LH.sub.N fragment of a Clostridial neurotoxin, for example botulinum toxin type A, is covalently coupled, using a spacer component to a targeting component forming an agent of the present invention. In another embodiment of the invention, the H.sub.c part of the Clostridial neurotoxin, for example botulinum toxin type A, may be mutated or modified, e.g. by chemical modification, to reduce, or preferably incapacitate, its ability to bind the neurotoxin to receptors at the neuromuscular junction. This modified Clostridial neurotoxin, for example botulinum toxin type A, is then covalently coupled, using one or more spacer components, to a targeting component forming an agent of the present invention. In one embodiment, a linker may be employed to join various components together. For example, a linker may be used to join a spacer component to a therapeutic component. Additionally, a linker may be used to join a therapeutic component with a targeting component. Various non-limiting embodiments which include the use of linkers are provided in the examples below.

Detailed Description Text (73):

According to another broad aspect of this invention recombinant techniques are used to produce at least one of the components of the agents. See, for example the disclosure of which is incorporated in its entirety herein by reference. The technique includes steps of obtaining genetic materials from DNA cloned from natural sources, or synthetic oligonucleotide sequences, which have codes for one of the components, for example the therapeutic, translocation and/or targeting component(s). The genetic constructs are incorporated into host cells for amplification by first fusing the genetic constructs with a cloning vectors, such as phages or plasmids. Then the cloning vectors are inserted into hosts, preferably *E. coli*'s. Following the expressions of the recombinant genes in host cells, the resultant proteins can be isolated using conventional techniques. The protein expressed may comprise all three components of the agent. For example, the protein expressed may include a light chain of botulinum toxin type A (the therapeutic component), a heavy chain, preferably the H.sub.N, of a botulinum toxin type A (the translocation component), and a Fab portion of an antibody which selectively binds to an alpha-2B adrenergic receptor under physiological conditions. In one embodiment, the protein expressed may include less than all three components of the agent. In such case, the components may be chemically joined, preferably through a spacer region.

Detailed Description Text (74):

There are many advantages to producing these agents recombinantly. For example, production of neurotoxin from anaerobic clostridium cultures is a cumbersome and time-consuming process including a multi-step purification protocol involving several protein precipitation steps and either prolonged and repeated crystallization of the toxin or several stages of column chromatography. Significantly, the high toxicity of the product dictates that the procedure must be performed under strict containment (BL-3). During the fermentation process, the

folded single-chain neurotoxins are activated by endogenous Clostridial proteases through a process termed nicking. This involves the removal of approximately 10 amino acid residues from the single-chain to create the dichain form in which the two chains remain covalently linked through the intrachain disulfide bond.

Detailed Description Text (75):

The nicked neurotoxin is more active than the unnicked form. The amount and precise location of nicking varies with the serotypes of the bacteria producing the toxin or with the modification made in the outer loop. The differences in single-chain neurotoxin activation and, hence, the yield of nicked toxin, are due to variations in the type and amounts of proteolytic 1 Is activity produced by a given strain. For example, greater than 99% of Clostridial botulinum type A single-chain neurotoxin is activated by the Hall A Clostridial botulinum strain, whereas type B and E strains produce toxins with lower amounts of activation (0 to 75% depending upon the fermentation time). Thus, the high toxicity of the mature neurotoxin plays a major part in the commercial manufacture of neurotoxins as therapeutic agents.

Detailed Description Text (76):

The degree of activation of engineered Clostridial toxins is, therefore, an important consideration for manufacture of these materials. It would be a major advantage if neurotoxins such as botulinum toxin and tetanus toxin could be expressed, recombinantly, in high yield in rapidly-growing bacteria (such as heterologous E. coli cells) as relatively non-toxic single-chains (or single chains having reduced toxic activity) which are safe, easy to isolate and simple to convert to the fully-active form.

Detailed Description Text (77):

With safety being a prime concern, previous work has concentrated on the expression in E. coli and purification of individual H and L chains of tetanus and botulinum toxins; these isolated chains are, by themselves, non-toxic; see Li et al., Biochemistry 33:7014-7020 (1994); Zhou et al., Biochemistry 34:15175-15181 (1995), hereby incorporated by reference herein. Following the separate production of these peptide chains and under strictly controlled conditions the H and L subunits can be combined by oxidative disulphide linkage to form the neuromuscular di-chains.

Detailed Description Text (78):

In one embodiment, an agent comprising a therapeutic component and a translocation component is recombinantly produced as an unnicked single chain. See Dolly et al. U.S. Pat. No. 09/648,692, the disclosure of which is incorporated in its entirety by reference herein. In a preferred embodiment, the agent includes an amino acid sequence that is susceptible to specific cleavage in vitro following expression as a single chain. Such proteins may include clostridial neurotoxins and derivatives thereof, such as those proteins disclosed in U.S. Pat. No. 5,989,545 and International Patent Application WO95/32738, both incorporated by reference herein.

Detailed Description Text (79):

In one embodiment of the invention the protein comprises the functional domains of a clostridial neurotoxin H chain and some or all of the functions of a clostridial neurotoxin L chain in a single polypeptide chain, and having an inserted proteolytic cleavage site located between the H domain and the L domain by which the single chain protein may be cleaved to produce the individual chains, preferably covalently linked by a disulfide linkage. The proteolytic cleavage sites comprise amino acid sequences that are selectively recognized and cleaved by a specific enzyme.

Detailed Description Text (80):

In a preferred embodiment of the invention, the expressed single-chain proteins comprise the biologically active domains of the H chain and L chain of a clostridial neurotoxin. Scission at the internal proteolytic cleavage site

separating the chain domains thus results in the activation of a neurotoxin having full activity.

Detailed Description Text (81):

In another embodiment of the invention the single-chain proteins comprise a targeting component targeted to a cell receptor other than one borne by a motor neuron. Such a binding domain may specific bind to, for example, an alpha-2B and/or adrenergic receptor. The single-chain proteins will contain a translocation component similar to that of clostridial neurotoxins, and a therapeutic component. The therapeutic component may be a clostridial neurotoxin light chain, or may be a different therapeutic component such as an enzyme, a transcribable nucleotide sequence, growth factor, an antisense nucleotide sequence and the like.

Detailed Description Text (83):

To minimize the safety risk associated with handling neurotoxin, the agents, or toxins of the this aspect of the present invention are expressed as their low activity (or inactive) single-chain proforms, then, by a carefully controlled proteolytic reaction in vitro, they are activated, preferably to the same potency level as the native neurotoxin from which they were derived. To improve the efficiency and rate of proteolytic cleavage the engineered proteolytic cleavage sites can be designed to occur in a specially-designed loop between the H and L portions of the single amino acid chain that promotes accessibility of the protease to the holotoxin substrate.

Detailed Description Text (85):

In another aspect of the invention the interchain loop region of the C. botulinum subtype E neurotoxin, which is normally resistant to proteolytic nicking in the bacterium and mammals, is modified to include the inserted proteolytic cleavage site, and this loop region used as the interchain loop region in the single-chain toxin or modified toxin molecules of the present invention. It is believed that using the loop from C. botulinum subtype E will stabilize the unnicked toxin molecule in vivo, making it resistant to undesired cleavage until activated through the use of the selected protease.

Detailed Description Text (87):

In one embodiment, a method for treating pain comprises the step of administering to a mammal, preferably a human, a therapeutically effective amount of an agent according to this invention. Various non-limiting examples of the types of pain which may be treated in accordance with this invention are chronic pain, allodynic pain, visceral pain, neuropathic pain and referred pain. In a preferred embodiment, the agent to be administered includes a therapeutic component which comprises a light chain of botulinum toxin type A, a translocation component which comprises a fragment of the heavy chain of botulinum toxin type A which is able to facilitate the transfer of at least the light chain into the cytoplasm of the target cell, and a targeting component which is represented by the general Formula IV: ##STR27##

Detailed Description Text (89):

The dose of the agent to be administered depends on many factors. For example, the better each one of the components is able to perform its respective function, the lower the dose of the agent is required to obtain a desired therapeutic effect. One of ordinary skill will be able to readily determine the specific dose for each specific agent. For agents employing a natural, mutated or recombinant botulinum toxin a the therapeutic and translocation component, an effective dose of an agent to be administered may be about 1 U to about 500 U of the botulinum toxin. In a preferred embodiment, the administered agent comprises about 10 U to about 300 U of the botulinum toxin.

Detailed Description Text (92):

The following examples illustrate how a therapeutic component, for example a light chain of a Clostridial toxin, may be recombinantly produced and reassociated with a

translocation component, for example a heavy chain of a Clostridial toxin. The examples also illustrate how the various components of an agent according to this invention may be joined together.

Detailed Description Text (95):

Example 1 describes the methods to clone the polynucleotide sequence encoding the BoNT/A-L chain. The DNA sequence encoding the BoNT/A-L chain may be amplified by a PCR protocol that employs synthetic oligonucleotides having the sequences, 5'-AAAGGCCTTTTGTTAATAAACAA-3' (SEQ ID#3) and 5'-GGAATTCTTACTTATTGTATCCTTTA-3' (SEQ ID#4). Use of these primers allows the introduction of Stu I and EcoR I restriction sites into the 5' and 3' ends of the BoNT/A-L chain gene fragment, respectively. These restriction sites may be subsequently used to facilitate unidirectional subcloning of the amplification products. Additionally, these primers introduce a stop codon at the C-terminus of the L chain coding sequence. Chromosomal DNA from C. botulinum (strain 63 A) may serve as a template in the amplification reaction.

Detailed Description Text (100):

Expression of the Botulinum Toxin Type A-L (BoNt/A-L) Chain Fusion Proteins

Detailed Description Text (139):

In accordance with the invention, a therapeutic component, such as a light chain, may be attached to a targeting component. The light chain upon which the targeting component is to be attached may be free from other attachments or may already be attached to a translocation component. Many approaches are known for linking chemical compounds to protein chains. For example, a linker molecule may be used to separate the targeting component from the L chain peptide. It is known that 11 amino acids may be attached to the N-terminus of the TeTx-L chain without substantially affecting its functionality. For this reason, the N-terminal portion of either the botulinum toxin or tetanus toxin L chain will be used as the targeting component attachment point.

Detailed Description Text (148):

Once the targeting component has a linker attached, the following reaction can be used to link the targeting component to the light chain, for example the light chain of botulinum toxin type A. In this reaction, the light chain, preferably the light chain of botulinum toxin type A, has an accessible lysine group that is used as the attachment point for the targeting component. As discussed hereinabove, an extra amino acid, such as lysine, can be readily added to the N-terminal portion of the L chain gene and used as the attachment point for a targeting component. In the following reaction, sodium cyanoborohydride is used to attach the linker to the lysine group on the L chain molecule.

CLAIMS:

4. An agent according to claim 2 wherein the light chain component comprises a light chain or a fragment thereof of a botulinum toxin, a butyricum toxin, a tetani toxin or biologically active variants thereof.
5. An agent according to claim 2 wherein the light chain component comprises a light chain or a fragment thereof of a botulinum toxin type A, B, C1, D, E, F, G or biologically active variants thereof.
6. An agent according to claim 2 wherein the light chain component comprises a light chain or a fragment thereof of a botulinum toxin type A or biologically active variants thereof.
10. An agent according to claim 9 wherein the translocation component facilitates the transfer of at least a part of the agent into a cytoplasm of the target cell.
11. An agent according to claim 9 wherein the translocation component facilitates

the transfer of the therapeutic component into a cytoplasm of the target cell.

13. An agent according to claim 12 wherein the heavy chain component comprises a heavy chain or a fragment thereof of a botulinum toxin, a butyricum toxin, a tetani toxin or biologically active variants thereof.

14. An agent according to claim 12 wherein the heavy chain component comprises a heavy chain or a fragment thereof of a botulinum toxin type A, B, Cl, D, E, F, G or biologically active variants thereof.

15. An agent according to claim 12 wherein the heavy chain component comprises a heavy chain or a fragment thereof of a botulinum toxin type A or biologically active active variants thereof.

17. An agent according to claim 9 wherein the therapeutic component comprises a light chain of a botulinum toxin type A and the translocation component comprises a fragment of a heavy chain of a botulinum toxin type A, wherein the fragment of a heavy chain can assist in the translocation of at least the therapeutic component into a cytoplasm of a cell.

33. An agent according to claim 32 wherein the therapeutic component is a light chain of a botulinum toxin type A, the translocation component is a fragment of a heavy chain of a botulinum toxin type A which can facilitate the translocation of at least the light chain into a cytoplasm of a cell, and the targeting component is represented by the formula: ##STR36## wherein X' is selected from the group consisting of R.sub.4 --C.dbd.C--R.sub.5 and R.sub.4 --C; a six membered carbon ring structure is formed when X' is R.sub.4 --C.dbd.C--R.sub.5 ; a five membered carbon ring is formed when X' is R.sub.4 --C; R.sub.1, R.sub.2, R.sub.3, R.sub.4 and R.sub.5 are each independently selected from the group consisting of F, Cl, Br, I, OR.sub.6 and H, wherein R.sub.6 is H or an alkyl, including a methyl, an ethyl or a propyl.

39. A method according to claim 38 wherein the therapeutic component comprises a light chain of botulium toxin type A and the translocation component comprises a fragment of a heavy chain which is able to facilitate the transfer of at least the light chain into a cytoplasm of the target cell.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	References	Claims	KWIC	Draw. Data
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L5: Entry 2 of 19

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DOCUMENT-IDENTIFIER: US 6776990 B2

TITLE: Methods and compositions for the treatment of pancreatitis

Brief Summary Text (15):

In neurons, neurotransmitters are packaged within synaptic vesicles, formed within the cytoplasm, then transported to the inner plasma membrane where the vesicles dock and fuse with the plasma membrane. Recent studies of nerve cells employing clostridial neurotoxins as probes of membrane fusion have revealed that fusion of synaptic vesicles with the cell membrane in nerve cells depends upon the presence of specific proteins that are associated with either the vesicle or the target

membrane. See id. These proteins have been termed SNAREs. As discussed in further detail below, a protein alternatively termed synaptobrevin or VAMP (vesicle-associated membrane protein) is a vesicle-associated SNARE (v-SNARE). There are at least two isoforms of synaptobrevin; these two isoforms are differentially expressed in the mammalian central nervous system, and are selectively associated with synaptic vesicles in neurons and secretory organelles in neuroendocrine cells. The target membrane-associated SNAREs (t-SNAREs) include syntaxin and SNAP-25. Following docking, the VAMP protein forms a core complex with syntaxin and SNAP-25; the formation of the core complex appears to be an essential step to membrane fusion. See Rizo & Sudhof, id. and Neimann et al., Trends in Cell Biol. 4:179-185 (May 1994), hereby incorporated by referenced herein.

Brief Summary Text (18):

Intoxication of neural cells by clostridial neurotoxins exploits specific characteristics of the SNARE proteins. These neurotoxins, most commonly found expressed in Clostridium botulinum and Clostridium tetanus, are highly potent and specific poisons of neural cells. These Gram positive bacteria secrete two related but distinct toxins, each comprising two disulfide-linked amino acid chains: a light chain (L) of about 50 KDa and a heavy chain (H) of about 100 KDa, which are wholly responsible for the symptoms of botulism and tetanus, respectively.

Brief Summary Text (19):

The tetanus and botulinum toxins are among the most lethal substances known to man; both toxins function by inhibiting neurotransmitter release in affected neurons. The tetanus neurotoxin (TeNT) acts mainly in the central nervous system, while botulinum neurotoxin (BoNT) acts at the neuromuscular junction; both toxins inhibit acetylcholine release from the nerve terminal of the affected neuron into the synapse, resulting in paralysis or reduced target organ function.

Brief Summary Text (20):

The tetanus neurotoxin (TeNT) is known to exist in one immunologically distinct type; the botulinum neurotoxins (BoNT) are known to occur in seven different immunologically distinct serotypes, termed BoNT/A through BoNT/G. While all of these latter types are produced by isolates of C. botulinum, two other species, C. baratii and C. butyricum also produce toxins similar to /F and /E, respectively. See e.g., Coffield et al., The Site and Mechanism of Action of Botulinum Neurotoxin in Therapy with Botulinum Toxin 3-13 (Jankovic J. & Hallett M. eds. 1994), the disclosure of which is incorporated herein by reference.

Brief Summary Text (21):

Regardless of type, the molecular mechanism of intoxication appears to be similar. In the first step of the process, the toxin binds to the presynaptic membrane of the target neuron through a specific interaction between the heavy chain and a neuronal cell surface receptor; the receptor is thought to be different for each type of botulinum toxin and for TeNT. The carboxy terminal (C-terminal) half of the heavy chain is required for targeting of the toxin to the cell surface. The cell surface receptors, while not yet conclusively identified, appear to be distinct for each neurotoxin serotype.

Brief Summary Text (23):

Either during or after translocation the disulfide bond joining the heavy and light chain is reduced, and the light chain is released into the cytoplasm. The entire toxic activity of botulinum and tetanus toxins is contained in the light chain of the holotoxin; the light chain is a zinc (Zn++) endopeptidase which selectively cleaves the SNARE proteins essential for recognition and docking of neurotransmitter-containing vesicles with the cytoplasmic surface of the plasma membrane, and fusion of the vesicles with the plasma membrane. The light chain of TxNT, BoNT/B, BoNT/D, BoNT/F, and BoNT/G cause specific proteolysis of VAMP, an integral protein. During proteolysis, most of the VAMP present at the cytosolic surface of the synaptic vesicle is inactivated as a result of any one of these

cleavage events. Each toxin cleaves a different specific peptide bond.

Brief Summary Text (24):

BoNT/A and /E selectively cleave the plasma membrane-associated SNARE protein SNAP-25; this protein is bound to and present on the cytoplasmic surface of the plasma membrane. BoNT/C1 cleaves syntaxin, which exists as an integral protein having most of its mass exposed to the cytosol. Syntaxin interacts with the calcium channels at presynaptic terminal active zones. See Tonello et al., Tetanus and Botulism Neurotoxins in Intracellular Protein Catabolism 251-260 (Suzuki K & Bond J. eds. 1996), the disclosure of which is incorporated by reference as part of this specification. Bo/NTC1 also appears to cleave SNAP-25.

Brief Summary Text (27):

International Patent Publication No. WO 96/33273 relates to derivatives of botulinum toxin designed to prevent neurotransmitter release from sensory afferent neurons to treat chronic pain. Such derivatives are targeted to nociceptive neurons using a targeting moiety that binds to a binding site of the surface of the neuron.

Brief Summary Text (31):

In one embodiment of this aspect, the invention is a therapeutic agent comprising a chimeric protein containing an amino acid sequence-specific endopeptidase activity which will specifically cleave at least one synaptic vesicle-associated protein selected from the group consisting of SNAP-25, syntaxin or VAMP, in combination with the translocation activity of the N-terminus of a clostridial neurotoxin heavy chain, wherein the chimeric protein further comprises a recognition domain which will bind a human cholecystokinin (CCK) receptor. Upon binding of the recognition domain of the protein to the CCK receptor, the protein is specifically transported into cells containing CCK receptors (pancreatic acinar cells) through receptor-mediated endocytosis. In a preferred embodiment, the CCK receptor is the CCK A receptor.

Brief Summary Text (32):

Once inside the acinar cell, the chimeric protein functions in a manner similar to that of a clostridial neurotoxin within its target neuron. The toxin moiety is translocated from the endosome into the cytoplasm, where it acts to cleave a SNARE protein identical or homologous to SNAP-25, syntaxin or VAMP. The cleavage of this protein prevents formation of a core complex between the SNARE proteins and thus prevents or reduces the extent of fusion of the vesicle with the target membrane. This, in turn, results in inhibition of zymogen release from the acinar cells and of zymogen activation by lysosomal hydrolases. The autodigestion of pancreatic tissue in acute pancreatitis is therefore reduced or eliminated.

Brief Summary Text (34):

Another embodiment of the invention concerns a therapeutic composition that contains the translocation activity of a clostridial neurotoxin heavy chain in combination with a recognition domain able to bind a specific cell type and a therapeutic element having an activity other than the endopeptidase activity of a clostridial neurotoxin light chain. A non-exclusive list of certain such therapeutic elements includes: hormones and hormone-agonists and antagonists, nucleic acids capable of being used as replication, transcription, or translational templates (e.g., for expression of a protein drug having the desired biological activity or for synthesis of a nucleic acid drug as an antisense agent), enzymes, toxins, and the like.

Brief Summary Text (36):

Another embodiment is drawn to methods for the treatment of acute pancreatitis comprising contacting an acinar cell with an effective amount of a composition comprising a chimeric protein containing an amino acid sequence-specific endopeptidase activity which will specifically cleave at least one synaptic

vesicle-associated protein selected from the group consisting of SNAP-25, syntaxin or VAMP, in combination with the translocation activity of the N-terminus of a clostridial neurotoxin heavy chain, wherein the chimeric protein further comprises a recognition domain able to bind to a cell surface protein characteristic of an human pancreatic acinar cell. Preferably the cell surface protein is a CCK receptor protein; most preferably the protein is the human CCK A protein. CCK receptors (CCK-A receptor and CCK-B receptor) are found mainly in on the surface of pancreatic acinar cells, although they are also found in some brain cells and, to a lesser extent on the surface of gastrointestinal cells.

Brief Summary Text (37):

Any suitable route of administration may be used in this aspect of the invention. Applicants currently prefer to administer the therapeutic agent in an intravenous infusion solution; however methods such as ingestion (particularly when associated with neurotoxin-associated proteins (NAPs); see Sharma et al., J. Nat. Toxins 7:239-7:239-253(1998), incorporated by reference herein), direct delivery to the pancreas, injection and the like may also be used. The agent is substantially specifically targeted to pancreatic cells; when the agent contains a CCK receptor-binding domain, the blood-brain barrier prevents the agent from interacting with brain cells.

Brief Summary Text (38):

In yet another embodiment the invention provides a composition comprising a drug or other therapeutic agent having an activity other than that of a clostridial neurotoxin light chain for intracellular delivery, said agent joined to the translocation domain of a clostridial neurotoxin heavy chain and a binding element able to recognize a cell surface receptor of a target cell. In a preferred embodiment, the target cell is not a neuron. Also, in this embodiment it is preferred that the drug or other therapeutic agent has an enzymatic, catalytic, or other self-perpetuating mode of activity, so that the effective dose of drug is greater than the number of drug molecules delivered within the target cell. A non-exclusive list of certain such drugs would include: hormones and hormone-agonists and antagonists, nucleic acids capable being of being used as replication, transcription, or translational templates (e.g., for expression of a protein drug having the desired biological activity or for synthesis of a nucleic acid drug as an antisense agent), enzymes, toxins (such as diphtheria toxin or ricin), and the like.

Detailed Description Text (26):

Such structural models provide detailed guidance to the person of ordinary skill in the art as to the construction of a variety of binding elements able to retain the binding characteristics of biologically active CCK peptides for the CCK-A receptor, for example, as, for example, by site directed mutagenesis of a clostridial neurotoxin heavy chain. Similarly, models deduced using similar methodologies have been proposed for the CCK B receptor, see e.g., Jagerschmidt, A. et al., Mol. Pharmacol. 48:783-789 (1995), and can be used as a basis for the construction of binding elements that retain binding characteristics similar to the CCK B receptor.

Detailed Description Text (29):

The translocation element comprises a portion of a clostridial neurotoxin heavy chain having a translocation activity. By "translocation" is meant the ability to facilitate the transport of a polypeptide through a vesicular membrane, thereby exposing some or all of the polypeptide to the cytoplasm.

Detailed Description Text (30):

In the various botulinum neurotoxins translocation is thought to involve an allosteric conformational change of the heavy chain caused by a decrease in pH within the endosome.

Detailed Description Text (32):

The amino acid sequence of the translocation-mediating portion of the botulinum neurotoxin heavy chain is known to those of skill in the art; additionally, those amino acid residues within this portion that are known to be essential for conferring the translocation activity are also known.

Detailed Description Text (33):

It would therefore be well within the ability of one of ordinary skill in the art, for example, to employ the naturally occurring N-terminal peptide half of the heavy chain of any of the various Clostridium tetanus or Clostridium botulinum neurotoxin subtypes as a translocation element, or to design an analogous translocation element by aligning the primary sequences of the N-terminal halves of the various heavy chains and selecting a consensus primary translocation sequence based on conserved amino acid, polarity, steric and hydrophobicity characteristics between the sequences.

Detailed Description Text (35):

In a preferred embodiment, the therapeutic element is a polypeptide comprising a clostridial neurotoxin light chain or a portion thereof retaining the SNARE-protein sequence-specific endopeptidase activity of a clostridial neurotoxin light chain. The amino acid sequences of the light chain of botulinum neurotoxin (BoNT) subtypes A-G have been determined, as has the amino acid sequence of the light chain of the tetanus neurotoxin (TeNT). Each chain contains the Zn.sup.++ -binding motif His-Glu-Glu-x-x-His (N terminal direction at the left) characteristic of Zn.sup.++ -dependent endopeptidases (HELIH in TeNT, BoNT/A /B and /E; HELNH in BoNT/C; and HELTH in BoNT/D).

Detailed Description Text (39):

The substrate specificities of the various clostridial neurotoxin light chains other than BoNT/A are known. Therefore, the person of ordinary skill in the art could easily determine the toxin residues essential in these subtypes for cleavage and substrate recognition (for example, by site-directed mutagenesis or deletion of various regions of the toxin molecule followed by testing of proteolytic activity and substrate specificity), and could therefore easily design variants of the native neurotoxin light chain that retain the same or similar activity.

Detailed Description Text (40):

Additionally, construction of the therapeutic agents set forth in this specification would be easily constructed by the person of skill in the art. It is well known that the clostridial neurotoxins have three functional domains analogous to the three elements of the present invention. For example, the BoNT/A neurotoxin light chain is present in amino acid residues 1-448 of the BoNT/A prototoxin (i.e., before nicking of the prototoxin to form the disulfide-linked dichain holotoxin); this amino acid sequence is provided below as SEQ ID NO: 7. Active site residues are underlined:

Detailed Description Text (44):

The amino acid sequence of the BoNT/A prototoxin is encoded by nucleotides 358 to 4245 of the neurotoxin cDNA sequence, set forth herein below as SEQ ID NO: 10.

Detailed Description Text (45):

Of course, three distinct domains analogous to those described above for BoNT/A exist for all the BoNT subtypes as well as for TeNT neurotoxin; an alignment of the amino acid sequences of these holotoxins will reveal the sequence coordinates for these other neurotoxin species.

Detailed Description Text (51):

A culture of Clostridium botulinum is permitted to grown to confluence. The cells are then lysed and total RNA is extracted according to conventional methods and in the presence of an RNase inhibitor. The RNA preparation is then passed over a oligo

(dT) cellulose column, the polyadenylated messenger RNA is permitted to bind, and the column is washed with 5-10 column volumes of 20 mM Tris pH 7.6, 0.5 M NaCl, 1 mM EDTA (ethylenediamine tetraacetic acid), 0.1% (w/v) SDS (sodium dodecyl sulfate). Polyadenylated RNA is then eluted with 2-3 column volumes of STE (10 mM Tris (pH 7.6), 1 mM EDTA, 0.05% (w/v) SDS). The pooled mRNA is then precipitated in 2 volumes of ice cold ethanol, pelleted in a centrifuge at 10,000.times.g for 15 minutes, then redissolved in a small volume of STE.

Detailed Description Text (52):

The BoNT/A mRNA is used as a template for DNA synthesis using Moloney murine leukemia virus reverse transcriptase (MMLV-RT), then the L chain and then H.sub.N chain of the neurotoxin is amplified from the cDNA by the polymerase chain reaction (PCR) using appropriate oligonucleotide primers whose sequences are designed based on the BoNT/A neurotoxin CDNA sequence of SEQ ID NO: 9. These procedures are performed using the standard techniques of molecular biology as detailed in, for example, Sambrook et al., already incorporated by reference herein. The primer defining the beginning of the coding region (5' side of the L chain fragment) is given a StuI site. The PCR primer defining the 3' end of the H.sub.N -encoding domain has the following features (from 3' to 5'): a 5' region sufficiently complementary to the 3' end of the H.sub.N -encoding domain to anneal thereto under amplification conditions, a nucleotide sequence encoding the human immunoglobulin hinge region .gamma..sub.1 (SEQ ID NO:11), a nucleotide sequence encoding the human CCK-8 octapeptide (SEQ ID NO:6), and a unique restriction endonuclease cleavage site.

Detailed Description Paragraph Table (5):

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Sharma et al, "Functional role of Hn-33: Enhanced cleavage of synaptic protein SNAP-25 by botulinum neurotoxin A and E"; Book of Abstracts, 216.

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Other Reference Publication (23):

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Other Reference Publication (25):

Binz et al, "The complete sequence of the botulinum type A neurotoxin and its comparison with other Clostridial neurotoxins", Gen Bank M30196, Web Site ncib.nlm.nih.gov.

CLAIMS:

1. A composition able to treat acute pancreatitis in a mammal comprising, a. a first element comprising a binding element selected from the group consisting of i) a first peptide comprising an amino acid sequence consisting of SEQ ID NO. 2 or a contiguous fragment thereof containing at least the 8 C-terminal residues of such region, wherein the C-terminal phenylalanine is amidated and/or the aspartic acid residue 7 amino acids from the C-terminus thereof is sulfated, and ii) said first peptide wherein said phenylalanine and aspartic acid residue have not been modified, and wherein said binding element is able to specifically bind a CCK-A or CCK-B receptor under physiological conditions, b. a second element comprising a translocation element derived from a Clostridial neurotoxin able to facilitate the transfer of a polypeptide across a vesicular membrane in a pancreatic cell, and c. a third element, linked to and comprised in a separate polypeptide chain from said first and second elements, comprising a therapeutic element derived from a Clostridial neurotoxin able, when present in the cytoplasm of a pancreatic cell, to inhibit or block enzymatic secretion by said pancreatic cell, and wherein following binding of said first element to a pancreatic acinar cell said third element is transported across a pancreatic cell membrane.

13. The composition of claim 1 wherein said composition further comprises a spacer moiety separating said binding element from said translocation element.

17. The composition of claim 7 wherein said composition further comprises a spacer moiety separating said binding element from said translocation element.

21. The composition of claim 8 wherein said composition further comprises a spacer moiety separating said binding element from said translocation element.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Abstract	Claims	KWC	Draw. Data
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☐ 3. Document ID: US 6682744 B1

L5: Entry 3 of 19

File: USPT

Jan 27, 2004

DOCUMENT-IDENTIFIER: US 6682744 B1

TITLE: Pro-gut maturation and anti-inflammatory effects of lactobacillus and lactobacillus secreted proteins, carbohydrates and lipids

Brief Summary Text (13):

A variety of in vitro studies indicate that endogenous intestinal bacteria can inhibit pathogenic bacteria. For example, Sullivan et al., Inhibitions of growth of C. botulinum by intestinal microflora isolated from healthy infants, Microbial Ecology in Health and Disease, 1:179-192 (1988), showed that gut isolates of Bfidobacteria, Lactobacilli, Proprionibacteria and Enterococci inhibit C. botulinum in vitro. Numerous in vivo studies also lend support to the ability of selected Lactobacilli to modify the intestinal microflora. Conway, Lactobacilli: Fact and fiction, Ch. 16 in The regulatory and protective role of the normal flora, Grun, Midvedt and Norin, eds., Stockton Press, pp. 263-281 (1988).

Other Reference Publication (8):

Sullivan et al., "Inhibition of Growth of Clostridium Botulinum by Intestinal Microflora Isolated from Healthy Infants," Microbial Ecology in Health and Disease, vol. 1, pp. 179-192, (1988).

## CLAIMS:

1. A method of reducing adherence or blocking translocation of bacteria, which are capable of causing necrotizing tissue injury in the mucosal cells of the gastrointestinal tract of a mammalian subject, said method comprising contacting mucosal cells of said mammalian host with cells and secretions of a Lactobacillus, said Lactobacillus being selected from the group consisting of Lactobacillus plantarum, deposited as ATCC 202195, and Lactobacillus salivarius, deposited as ATCC 202196.

3. A method of reducing adherence or blocking translocation of bacteria into mucosal cells of the gastrointestinal tract of a mammalian subject, said bacteria being capable of causing neonatal necrotizing enterocolitis, said method comprising contacting mucosal cells of said mammalian subject with cells and secretions of a Lactobacillus, said Lactobacillus being selected from the group consisting of Lactobacillus plantarum, deposited as ATCC 202195, and Lactobacillus salivarius, deposited as ATCC 202196.

4. A method of reducing adherence or blocking translocation of bacteria into the mucosal cells of the gastrointestinal tract of a mammalian subject, said bacteria being capable of causing gastrointestinal dysfunction in a said mammalian subject, said dysfunction characterized by infection or inflammation, said method comprising contacting mucosal cells of said mammalian subject with cells and secretions of a Lactobacillus, said Lactobacillus being selected from the group consisting of Lactobacillus plantarum, deposited as ATCC 202195, and Lactobacillus salivarius, deposited as ATCC 202196.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Abstracts	Claims	KWIC	Draw. De
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☐ 4. Document ID: US 6632440 B1

L5: Entry 4 of 19

File: USPT

Oct 14, 2003

DOCUMENT-IDENTIFIER: US 6632440 B1

TITLE: Methods and compounds for the treatment of mucus hypersecretion

Brief Summary Text (14):

A compound of embodiments of the invention is a polypeptide that consists of or comprises an inhibiting domain which inhibits exocytosis in the mucus secreting cell or inhibits exocytosis in a neuronal cell, thereby directly inhibiting exocytosis of mucus or one or more mucus components or indirectly inhibiting mucus secretion by inhibiting exocytosis of neurotransmitter which would in turn lead to or otherwise stimulate mucus secretion. The inhibiting domain can suitably comprise a light chain of a clostridial neurotoxin, or a fragment or variant thereof which inhibits exocytosis.

Brief Summary Text (15):

The compound preferably further comprises a translocating domain that translocates the inhibiting domain into the cell. This domain may comprise a H.sub.N region of a botulinum polypeptide, or a fragment or variant thereof that translocates the inhibiting domain into the cell.

Brief Summary Text (18):

The compound of specific embodiments of the invention comprises first, second and third domains. The first domain is adapted to cleave one or more vesicle or plasma-membrane associated proteins essential to exocytosis. This domain prevents exocytosis once delivered to a targeted cell. The second domain translocates the compound into the cell. This domain delivers the first domain into the cell. The third domain binds to the target cell, ie binds to (i) a mucus secreting cell, or (ii) a neuronal cell controlling or directing mucus secretion, and may be referred to as a targeting moiety ("TM"). The compound may be derived from a toxin and it is preferred that such a compound is free of clostridial neurotoxin and free of any clostridial neurotoxin precursor that can be converted into toxin. Botulinum and tetanus toxin are suitable sources of domains for the compounds of the invention.

Brief Summary Text (20):

Surprisingly, agents of the present invention for treatment of mucus hypersecretion can be produced by modifying a clostridial neurotoxin or fragment thereof. The clostridial neurotoxins share a common architecture of a catalytic L-chain (LC, ca 50 kDa) disulphide linked to a receptor binding and translocating H-chain (HC, ca 100 kDa). The HC polypeptide is considered to comprise all or part of two distinct functional domains. The carboxy-terminal half of the HC (ca 50 kDa), termed the H.sub.c domain, is involved in the high affinity, neurospecific binding of the neurotoxin to cell surface receptors on the target neuron, whilst the amino-terminal half, termed the H.sub.N domain (ca 50 kDa), is considered to mediate the translocation of at least some portion of the neurotoxin across cellular membranes such that the functional activity of the LC is expressed within the target cell. The H.sub.N domain also has the property, under conditions of low pH, of forming ion-permeable channels in lipid membranes, this may in some manner relate to its translocation function.

Brief Summary Text (21):

For botulinum neurotoxin type A (BoNT/A) these domains are considered to reside within amino acid residues 872-1296 for the H.sub.c, amino acid residues 449-871 for the H.sub.N and residues 1-448 for the LC. Digestion with trypsin effectively degrades the H.sub.c domain of the BoNT/A to generate a non-toxic fragment

designated LH.sub.N, which is no longer able to bind to and enter neurons. The LH.sub.N fragment so produced also has the property of enhanced solubility compared to both the parent holotoxin and the isolated LC.

Brief Summary Text (22):

It is therefore possible to provide functional definitions of the domains within the neurotoxin molecule, as follows: (A) clostridial neurotoxin light chain: A metalloprotease exhibiting high substrate specificity for vesicle and/or plasma membrane associated proteins involved in the exocytotic process.

Brief Summary Text (23):

In particular, it cleaves one or more of SNAP-25, VAMP (synaptobrevin/cellubrevin) and syntaxin. (B) clostridial neurotoxin heavy chain H.sub.N domain: A portion of the heavy chain which enables translocation of that portion of the neurotoxin molecule such that a functional expression of light chain activity occurs within a target cell. The domain responsible for translocation of the endopeptidase activity, following binding of neurotoxin to its specific cell surface receptor via the binding domain, into the target cell. The domain responsible for formation of ion-permeable pores in lipid membranes under conditions of low pH. The domain responsible for increasing the solubility of the entire polypeptide compared to the solubility of light chain alone. (C) clostridial neurotoxin heavy chain H.sub.c domain.

Brief Summary Text (24):

A portion of the heavy chain which is responsible for binding of the native holotoxin to cell surface receptor(s) involved in the intoxicating action of clostridial toxin prior to internalisation of the toxin into the cell.

Brief Summary Text (25):

The identity of the cellular recognition markers for these toxins is currently not understood and no specific receptor species have yet been identified although Kozaki et al. have reported that synaptotagmin may be the receptor for botulinum neurotoxin type B. It is probable that each of the neurotoxins has a different receptor.

Brief Summary Text (26):

By covalently linking a clostridial neurotoxin, or a hybrid of two clostridial neurotoxins, in which the H.sub.c region of the H-chain has been removed or modified, to a new molecule or moiety, the Targeting Moiety (TM), that binds to a BS on the surface of the relevant secretory cells and or neurones in the airways responsible for secretion of mucus and or regulation of said secretion, a novel agent capable of inhibiting mucus secretion is produced. A further surprising aspect of the present invention is that if the L-chain of a clostridial neurotoxin, or a fragment of the L-chain containing the endopeptidase activity, is covalently linked to TM which can also effect internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant secretory cells and or neurones in the airways responsible for secretion of mucus and or regulation of said secretion, this also produces a novel agent capable of inhibiting mucus secretion.

Brief Summary Text (27):

Accordingly, the invention may thus provide a compound containing a first domain equivalent to a clostridial toxin light chain and a second domain providing the functional aspects of the H.sub.N of a clostridial toxin heavy chain, whilst lacking the functional aspects of a clostridial toxin H.sub.c domain, and a third domain which binds to the target mucus secreting or mucus secretion controlling cell.

Brief Summary Text (28):

For the purposes of the invention, the functional property or properties of the

H.sub.N of a clostridial toxin heavy chain that are to be exhibited by the second domain of the polypeptide of the invention is translocation of the first domain into a target cell once the compound is proximal to the target cell. References hereafter to a H.sub.N domain or to the functions of a H.sub.N domain are references to this property or properties. The second domain is not required to exhibit other properties of the H.sub.N domain of a clostridial toxin heavy chain. A second domain of the invention can thus be relatively insoluble but retain the translocation function of a native toxin--this is of use if solubility is not essential to its administration or if necessary solubility is imparted to a composition made up of that domain and one or more other components by one or more of said other components.

Brief Summary Text (30):

The first domain optionally comprises a fragment or variant of a clostridial toxin light chain. The fragment is optionally an N-terminal, or C-terminal fragment of the light chain, or is an internal fragment, so long as it substantially retains the ability to cleave the vesicle or plasma-membrane associated protein essential to exocytosis. Domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem., Vol.267, No. 21, July 1992, pages 14721-14729. The variant has a different peptide sequence from the light chain or from the fragment, though it too is capable of cleaving the vesicle or plasma-membrane associated protein. It is conveniently obtained by insertion, deletion and/or substitution of a light chain or fragment thereof. In embodiments of the invention described below a variant sequence comprises (i) an N-terminal extension to a clostridial toxin light chain or fragment (ii) a clostridial toxin light chain or fragment modified by alteration of at least one amino acid (iii) a C-terminal extension to a clostridial toxin light chain or fragment, or (iv) combinations of 2 or more of (i)-(iii).

Brief Summary Text (31):

In an embodiment of the invention described in an example below, the toxin light chain and the portion of the toxin heavy chain are of botulinum toxin type A. In a further embodiment of the invention described in an example below, the toxin light chain and the portion of the toxin heavy chain are of botulinum toxin type B. The polypeptide optionally comprises a light chain or fragment or variant of one toxin type and a heavy chain or fragment or variant of another toxin type.

Brief Summary Text (32):

In a polypeptide according to the invention said second domain preferably comprises a clostridial toxin heavy chain H.sub.N portion or a fragment or variant of a clostridial toxin heavy chain H.sub.N portion. The fragment is optionally an N-terminal or C-terminal or internal fragment, so long as it retains the function of the H.sub.N domain. Teachings of regions within the H.sub.N responsible for its function are provided for example in Biochemistry 1995, 34, pages 15175-15181 and Eur. J. Biochem, 1989, 185, pages 197-203. The variant has a different sequence from the H.sub.N domain or fragment, though it too retains the function of the H.sub.N domain. It is conveniently obtained by insertion, deletion and/or substitution of a H.sub.N domain or fragment thereof. In embodiments of the invention, described below, it comprises (i) an N-terminal extension to a H.sub.N domain or fragment, (ii) a C-terminal extension to a H.sub.N domain or fragment, (iii) a modification to a H.sub.N domain or fragment by alteration of at least one amino acid, or (iv) combinations of 2 or more of (i)-(iii). The clostridial toxin is preferably botulinum toxin or tetanus toxin.

Brief Summary Text (35):

It is known in the art that the H.sub.c portion of the neurotoxin molecule can be removed from the other portion of the H-chain, known as H.sub.N, such that the H.sub.N fragment remains disulphide linked to the L-chain of the neurotoxin providing a fragment known as LH.sub.N. Thus, in one embodiment of the present invention the LH.sub.N fragment of a clostridial neurotoxin is covalently linked,

using linkages which may include one or more spacer regions, to a TM.

Brief Summary Text (36):

The H.sub.c domain of a clostridial neurotoxin may be mutated or modified, eg by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction. This modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

Brief Summary Text (37):

The heavy chain of a clostridial neurotoxin, in which the H.sub.c domain is mutated or modified, eg by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction, may be combined with the L-chain of a different clostridial neurotoxin. This hybrid, modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

Brief Summary Text (38):

In another embodiment of the invention, the H.sub.N domain of a clostridial neurotoxin is combined with the L-chain of a different clostridial neurotoxin. This hybrid LH.sub.N is then covalently linked, using linkages which may include one or more spacer regions, to a TM. In a further embodiment of the invention, the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is covalently linked, using linkages which may include one or more spacer regions, to a TM which can also effect the internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant secretory and/or neuronal cells in the airways responsible for secretion of mucus and or regulation of said secretion.

Brief Summary Text (39):

The agent is optionally expressed recombinantly as a fusion protein which includes an appropriate TM in addition to any desired spacer regions. The recombinantly expressed agent may be derived wholly from the gene encoding one serotype of neurotoxin or may be a chimaera derived from genes encoding one or more serotypes. In another embodiment of the invention the required LH.sub.N, which may be a hybrid of an L and H.sub.N from different clostridial types, is expressed recombinantly as a fusion protein with the TM, and may include one or more spacer regions

Brief Summary Text (40):

The light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, may be expressed recombinantly as a fusion protein with a TM which can also effect the internalisation of the L-chain, or a fragment of the L-chain containing the endopeptidase activity, into the cytoplasm of the relevant secretory and or neuronal cells in the airways responsible for secretion of mucus and or regulation of said secretion. The expressed fusion protein may also include one or more spacer regions.

Brief Summary Text (41):

A neurotoxin fragment as described in the present invention can be prepared by methods well known in the protein art, including, but not limited to, proteolytic cleavage or by genetic engineering strategies. Said fragment is preferably a non-toxic fragment. The conjugation may be chemical in nature using chemical or covalent linkers. Conjugates according to the present invention may be prepared by conventional methods known in the art.

Brief Summary Text (45):

In a specific embodiment of the invention, described in further detail below, a polypeptide according to the invention comprises Substance P, and an L chain and a heavy chain H.sub.N region of botulinum toxin A. In use, this may be administered to a patient by aerosol. A solution of the polypeptide is prepared and converted

into an aerosol using a nebuliser for inhalation into the lungs of nebulised particles of diameter 1-5 microns.

Brief Summary Text (51):

The invention yet further provides a method of manufacture of a pharmaceutical composition, comprising: obtaining a clostridial neurotoxin and modifying it so as to remove or disable its H.sub.c portion; or obtaining a clostridial neurotoxin the H.sub.c portion of which has been removed or disabled; linking the toxin with a targeting moiety that binds to (i) a mucus secreting cell, or (ii) a neuronal cell that controls or directs mucus secretion.

Other Reference Publication (2):

Blaustein, R.O. et al., "The N-terminal half of the heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayers," FEBS Letters 226:115-120, Elsevier Science Publishers B.V. (Biomedical Division) (Dec. 1987).

Other Reference Publication (7):

Shone, C.C. et al., "A 50-kDa fragment from the NH.sub.2 -terminus of the heavy subunit of Clostridium botulinum type A neurotoxin forms channels in lipid vesicles," Eur. J. Biochem. 167:175-180, Springer International (Aug. 17, 1987).

Other Reference Publication (9):

Hambleton, P., "Clostridium botulinum toxins: a general review of involvement in disease, structure, mode of action and preparation for clinical use," J. Neurol. 239:16-20, Springer-Verlag (1992).

Other Reference Publication (10):

Kurazono, H., et al., "Minimal Essential Domains Specifying Toxicity of the Light Chains of Tetanus Toxin and Botulinum Neurotoxin Type A," J. Biol. Chem. 267:14721-14729, American Society for Biochemistry and Molecular Biology, Inc. (1992).

Other Reference Publication (11):

Nishiki, T., et al., "Identification of Protein Receptor for Clostridium botulinum Type B Neurotoxin in Rat Brain Synaptosomes," J. Biol. Chem. 269:10498-10503, American Society for Biochemistry and Molecular Biology, Inc. (1994).

Other Reference Publication (12):

Nishiki, T., et al., "The high-affinity binding of Clostridium botulinum type B neurotoxin to synaptotagmin II associated with gangliosides G.sub.T1b /G.sub.D1a," FEBS Lett. 378:253-257, Federation of European Biochemical Societies (1996).

Other Reference Publication (13):

Poulain, B., et al., "Inhibition of transmitter release by botulinum neurotoxin A. Contributions of various fragments to the intoxication process," Eur. J. Biochem. 185:197-203, Federation of European Biochemical Societies (1989).

Other Reference Publication (14):

Zhou, L., et al., "Expression and Purification of the Light Chain of Botulinum Neurotoxin A: A Single Mutation Abolishes Its Cleavage of SNAP-25 and Neurotoxicity after Reconstitution with the Heavy Chain," Biochem. 34:15175-15181, American Chemical Society (1995).

CLAIMS:

1. A method of treating hypersecretion of mucus, comprising administering, topically to the airways of a patient in need thereof, a therapeutically effective amount of a compound, said compound comprising: (a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to a target cell selected from the group consisting of (i) a mucus

mucus secreting cell, and (ii) a neuronal cell controlling or directing mucus secretion; and (c) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell; with the proviso that said compound is not a botulinum toxin; and wherein, following administration to said patient, the compound binds to and delivers the L-chain or L-chain fragment into said target cell, thereby (i) inhibiting mucus secretion by mucus secreting cells, (ii) inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion, or (iii) inhibiting mucus secretion by mucus secreting cells and inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

3. The method according to claim 1, wherein the translocating domain comprises the H.sub.N region of a botulinum toxin.

4. A compound which inhibits mucus secretion by mucus secreting cells, said compound comprising: (a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that selectively binds to a target cell that is a mucus secreting cell; and (c) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell; with the proviso that the compound is not a botulinum toxin.

6. The compound according to claim 4, wherein the translocating domain comprises the H.sub.N domain of a botulinum polypeptide.

7. A pharmaceutical composition, for topical administration to airways of a patient suffering from mucus hypersecretion, comprising: (a) an amount of a compound effective to inhibit mucus hypersecretion, wherein the compound comprises: (i) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (ii) a targeting domain that selectively binds to a target cell that is a mucus secreting cell; and (iii) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell, with the proviso that the compound is not a botulinum toxin; and (b) a formulation component selected from the group consisting of an excipient, an adjuvant and a propellant; wherein the composition is for nasal or oral administration of the compound to a patient.

9. A method for treating chronic obstructive pulmonary disease (COPD), comprising administering, topically to the airways of a patient in need thereof, a therapeutically effective amount of a compound, said compound comprising: (a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to a target cell selected from the group consisting of (i) a mucus secreting cell, and (ii) a neuronal cell controlling or directing mucus secretion; and (c) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell; with the proviso that the compound is not a botulinum toxin; and wherein following administration to said patient the compound binds to and delivers the L-chain or L-chain fragment into said target cell, thereby (i) inhibiting mucus secretion by mucus secreting cells, (ii) inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion, or (iii) inhibiting mucus secretion by mucus secreting cells and inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

10. A method for treating asthma, comprising administering, topically to the airways of a patient in need thereof, a therapeutically effective amount of a compound, said compound comprising: (a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active

proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to a target cell selected from the group consisting of (i) a mucus secreting cell, and (ii) a neuronal cell controlling or directing mucus secretion; and (c) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell; with the proviso that the compound is not a botulinum toxin; and wherein following administration to said patient the compound binds to and delivers the L-chain or L-chain fragment into said target cell, thereby (i) inhibiting mucus secretion by mucus secreting cells, (ii) inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion, or (iii) inhibiting mucus secretion by mucus secreting cells and inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

11. A method of manufacture of the compound according to claim 4, comprising: (a) obtaining a clostridial neurotoxin and removing or disabling the native target cell binding domain (Hc) of said clostridial neurotoxin to produce a modified clostridial neurotoxin; or (b) obtaining a modified clostridial neurotoxin that has had the native target cell binding domain (Hc) removed or disabled; and (c) linking the modified neurotoxin with a targeting domain that selectively binds the compound to (i) a mucus secreting cell, or (ii) a neuronal cell that controls or directs mucus secretion.

12. A method of manufacture of the compound according to claim 4, comprising linking together: (a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (b) a translocating domain that translocates the L-chain or L-chain fragment into the target cell; and (c) a targeting domain that selectively binds the compound to (i) a mucus secreting cell, or (ii) a neuronal cell that controls or directs mucus secretion.

14. A method of treating hypersecretion of mucus, comprising administering, topically to the airways of a patient in need thereof, a therapeutically effective amount of a compound, said compound comprising: (a) a light (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, serous cells, sensory efferent C-fibres, and Non-adrenal Non-Cholinergic neural system fibres; and (c) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell; with the proviso that the compound is not a botulinum toxin; and wherein, following administration to said patient, the compound binds to and delivers the L-chain or L-chain fragment into said target cell, thereby (i) inhibiting mucus secretion by mucus secreting cells, (ii) inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion, or (iii) inhibiting mucus secretion by mucus secreting cells and inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

15. A compound which inhibits mucus secretion by inhibiting mucus secretion by mucus secreting cells, said compound comprising: (a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that selectively binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and and serous cells; and (c) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell, with the proviso that the compound is not a botulinum toxin.

16. A compound which inhibits mucus secretion by inhibiting mucus secretion by mucus secreting cells, said compound comprising: (a) a light chain (L-chain) or L-

chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to (i) a mucus secreting cell, but not to (ii) a neuronal cell controlling or directing mucus secretion; and (c) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell, with the proviso that the compound is not a botulinum toxin.

18. The compound according to claim 16, wherein the translocating domain comprises a H.sub.N region of a botulinum toxin, or a fragment or variant thereof that translocates the L-chain or L-chain fragment into the cell.

19. A compound which inhibits mucus secretion by inhibiting mucus secretion by mucus secreting cells, said compound comprising: (a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment comprises the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to (i) a mucus secreting cell, but not to (ii) a neuronal cell controlling or directing mucus secretion, wherein said targeting domain binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and serous cells; and (c) a translocating domain of a clostridial neurotoxin that translocates the L-chain or L-chain fragment into the target cell, with the proviso that the compound is not a botulinum toxin.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstracts	References	Claims	KWIC	Draw. De
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☐ 5. Document ID: US 6506399 B2

L5: Entry 5 of 19

File: USPT

Jan 14, 2003

DOCUMENT-IDENTIFIER: US 6506399 B2

TITLE: Biodegradable botulinum toxin implant

Abstract Text (1):

A controlled release system for multiphasic, in vivo release of therapeutic amounts of botulinum toxin in a human patient over a prolonged period of time. The controlled release system can comprise a plurality of botulinum toxin incorporating polymeric microspheres.

Brief Summary Text (2):

The present invention relates to an implantable drug delivery system. In particular, the present invention relates to an implantable botulinum toxin delivery system.

Brief Summary Text (23):

Botulinum Toxin

Brief Summary Text (24):

The anaerobic, gram positive bacterium Clostridium botulinum produces a potent polypeptide neurotoxin, botulinum toxin, which causes a neuromuscular illness in humans and animals referred to as botulism. The spores of Clostridium botulinum are found in soil and can grow in improperly sterilized and sealed food containers of home based canneries, which are the cause of many of the cases of botulism. The effects of botulism typically appear 18 to 36 hours after eating the foodstuffs infected with a Clostridium botulinum culture or spores. The botulinum toxin can apparently pass unattenuated through the lining of the gut and attack peripheral

motor neurons. Symptoms of botulinum toxin intoxication can include nausea, difficulty walking and swallowing, and can progress to paralysis of respiratory muscles, cardiac failure and death.

Brief Summary Text (25):

Botulinum toxin type A is the most lethal natural biological agent known to man. About 50 picograms of a commercially available botulinum toxin type A (available from Allergan, Inc., Irvine, Calif. under the tradename BOTOX.RTM. (purified neurotoxin complex) in 100 unit vials) is a LD.sub.50 in mice (i.e. 1 unit). Thus, one unit of BOTOX.RTM. contains about 50 picograms (about 56 attomoles) of botulinum toxin type A complex. Interestingly, on a molar basis, botulinum toxin type A is about 1.8 billion times more lethal than diphtheria, about 600 million times more lethal than sodium cyanide, about 30 million times more lethal than cobra toxin and about 12 million times more lethal than cholera. Singh, Critical Aspects of Bacterial Protein Toxins, pages 63-84 (chapter 4) of Natural Toxins II, edited by B. R. Singh et al., Plenum Press, New York (1996) (where the stated LD.sub.50 of botulinum toxin type A of 0.3 ng equals 1 U is corrected for the fact that about 0.05 ng of BOTOX.RTM. equals 1 unit). One unit (U) of botulinum toxin is defined as the LD.sub.50 upon intraperitoneal injection into female Swiss Webster mice weighing 18 to 20 grams each.

Brief Summary Text (26):

Neurotransmitters are packaged in synaptic vesicles within the cytoplasm of neurons and are then transported to the inner plasma membrane where the vesicles dock and fuse with the plasma membrane. Recent studies of nerve cells employing clostridial neurotoxins as probes of membrane fusion have revealed that fusion of synaptic vesicles with the cell membrane in nerve cells depends upon the presence of specific proteins that are associated with either the vesicle or the target membrane. These proteins have been termed SNAREs. A protein alternatively termed synaptobrevin or VAMP (vesicle-associated membrane protein) is a vesicle-associated SNARE (v-SNARE). There are at least two isoforms of synaptobrevin; these two isoforms are differentially expressed in the mammalian central nervous system, and are selectively associated with synaptic vesicles in neurons and secretory organelles in neuroendocrine cells. The target membrane-associated SNAREs (t-SNAREs) include syntaxin and SNAP-25. Following docking, the VAMP protein forms a core complex with syntaxin and SNAP-25; the formation of the core complex appears to be an essential step to membrane fusion. See Neimann et al., Trends in Cell Biol. 4:179-185:1994

Brief Summary Text (27):

Seven generally immunologically distinct botulinum neurotoxins have been characterized, these being respectively botulinum neurotoxin serotypes A, B, C.sub.1, D, E, F and G each of which is distinguished by neutralization with type-specific antibodies. The different serotypes of botulinum toxin vary in the animal species that they affect and in the severity and duration of the paralysis they evoke. For example, it has been determined that botulinum toxin type A is 500 times more potent, as measured by the rate of Paralysis produced in the rat, than is botulinum toxin type B. Additionally, botulinum toxin type B has been determined to be non-toxic in primates at a dose of 480 U/kg which is about 12 times the primate LD.sub.50 for botulinum toxin type A. Botulinum toxin apparently binds with high affinity to cholinergic motor neurons, is translocated into the neuron and blocks the release of acetylcholine.

Brief Summary Text (28):

Regardless of serotype, the molecular mechanism of toxin intoxication appears to be similar and to involve at least three steps or stages. In the first step of the process, the toxin binds to the presynaptic membrane of the target neuron through a specific interaction between the heavy chain, H chain, and a cell surface receptor; the receptor is thought to be different for each type of botulinum toxin and for tetanus toxin. The carboxyl end segment of the H chain, H.sub.C, appears to be

important for targeting of the toxin to the cell surface.

Brief Summary Text (30):

The last step of the mechanism of botulinum toxin activity appears to involve reduction of the disulfide bond joining the heavy chain, H chain, and the light chain, L chain. The entire toxic activity of botulinum and tetanus toxins is contained in the L chain of the holotoxin; the L chain is a zinc (Zn++) endopeptidase which selectively cleaves proteins essential for recognition and docking of neurotransmitter-containing vesicles with the cytoplasmic surface of the plasma membrane, and fusion of the vesicles with the plasma membrane. Tetanus neurotoxin, and botulinum toxins B, D, F, and G cause degradation of synaptobrevin (also called vesicle-associated membrane protein (VAMP)), a synaptosomal membrane protein. Most of the VAMP present at the cytoplasmic surface of the synaptic vesicle is removed as a result of any one of these cleavage events. Serotype A and E cleave SNAP-25. Serotype C, was originally thought to cleave syntaxin, but was found to cleave syntaxin and SNAP-25. Each toxin specifically cleaves a different bond (except tetanus and type B which cleave the same bond).

Brief Summary Text (31):

Botulinum toxins have been used in clinical settings for the treatment of neuromuscular disorders characterized by hyperactive skeletal muscles. Botulinum toxin type A was approved by the U.S. Food and Drug Administration in 1989 for the treatment of blepharospasm, strabismus and hemifacial spasm. Non-type A botulinum toxin serotypes apparently have a lower potency and/or a shorter duration of activity as compared to botulinum toxin type A. Clinical effects of peripheral intramuscular botulinum toxin type A are usually seen within one week of injection. The typical duration of symptomatic relief from a single intramuscular injection of botulinum toxin type A averages about three months.

Brief Summary Text (32):

Although all the botulinum toxins serotypes apparently inhibit release of the neurotransmitter acetylcholine at the neuromuscular junction, they do so by affecting different neurosecretory proteins and/or cleaving these proteins at different sites. For example, botulinum types A and E both cleave the 25 kiloDalton (kD) synaptosomal associated protein (SNAP-25), but they target different amino acid sequences within this protein. Botulinum toxin types B, D, F and G act on vesicle-associated protein (VAMP, also called synaptobrevin), with each serotype cleaving the protein at a different site. Finally, botulinum toxin type C.sub.1 has been shown to cleave both syntaxin and SNAP-25. These differences in mechanism of action may affect the relative potency and/or duration of action of the various botulinum toxin serotypes. Apparently, a substrate for a botulinum toxin can be found in a variety of different cell types. See e.g. Biochem, J 1;339 (pt 1):159-65:1999, and Mov Disord, 10(3): 376:1995 (pancreatic islet B cells contain at least SNAP-25 and synaptobrevin).

Brief Summary Text (33):

The molecular weight of the botulinum toxin protein molecule, for all seven of the known botulinum toxin serotypes, is about 150 kD. Interestingly, the botulinum toxins are released by Clostridial bacterium as complexes comprising the 150 kD botulinum toxin protein molecule along with associated non-toxin proteins. Thus, the botulinum toxin type A complex can be produced by Clostridial bacterium as 900 kD, 500 kD and 300 kD forms. Botulinum toxin types B and C.sub.1 is apparently produced as only a 700 kD or 500 kD complex. Botulinum toxin type D is produced as both 300 kD and 500 kD complexes. Finally, botulinum toxin types E and F are produced as only approximately 300 kD complexes. The complexes (i.e. molecular weight greater than about 150 kD) are believed to contain a non-toxin hemagglutinin protein and a non-toxin and non-toxic nonhemagglutinin protein. These two non-toxin proteins (which along with the botulinum toxin molecule comprise the relevant neurotoxin complex) may act to provide stability against denaturation to the botulinum toxin molecule and protection against digestive acids when toxin is

ingested. Additionally, it is possible that the larger (greater than about 150 kD molecular weight) botulinum toxin complexes may result in a slower rate of diffusion of the botulinum toxin away from a site of intramuscular injection of a botulinum toxin complex.

Brief Summary Text (34):

In vitro studies have indicated that botulinum toxin inhibits potassium cation induced release of both acetylcholine and norepinephrine from primary cell cultures of brainstem tissue. Additionally, it has been reported that botulinum toxin inhibits the evoked release of both glycine and glutamate in primary cultures of spinal cord neurons and that in brain synaptosome preparations botulinum toxin inhibits the release of each of the neurotransmitters acetylcholine, dopamine, norepinephrine (Habermann E., et al., Tetanus Toxin and Botulinum A and C Neurotoxins Inhibit Noradrenaline Release From Cultured Mouse Brain, J Neurochem 51 (2);522-527:1988) CGRP, substance P and glutamate (Sanchez-Prieto, J., et al., Botulinum Toxin A Blocks Glutamate Exocytosis From Guinea Pig Cerebral Cortical Synaptosomes, Eur J. Biochem 165;675-681 :1987. Thus, when adequate concentrations are used, stimulus-evoked release of most neurotransmitters is blocked by botulinum toxin. See e.g. Pearce, L. B., Pharmacologic Characterization of Botulinum Toxin For Basic Science and Medicine, Toxicon 35(9); 1373-1412 at 1393 (1997); Bigalke H., et al., Botulinum A Neurotoxin Inhibits Non-Cholinergic Synaptic Transmission in in Mouse Spinal Cord Neurons in Culture, Brain Research 360;318-324:1985; Habermann E., Inhibition by Tetanus and Botulinum A Toxin of the Release of [.sup.3 H] Noradrenaline and [.sup.3 H]GABA From Rat Brain Homogenate, Experientia 44;224-226:1988, Bigalke H., et al., Tetanus Toxin and Botulinum A Toxin Inhibit Release and Uptake of Various Transmitters, as Studied with Particulate Preparations From Rat Brain and Spinal Cord, Naunyn-Schmiedeberg's Arch Pharmacol 316;244-251:1981, and; Jankovic J. et al., Therapy With Botulinum Toxin, Marcel Dekker, Inc., (1994), page 5.

Brief Summary Text (35):

Botulinum toxin type A can be obtained by establishing and growing cultures of Clostridium botulinum in a fermenter and then harvesting and purifying the fermented mixture in accordance with known procedures. All the botulinum toxin serotypes are initially synthesized as inactive single chain proteins which must be cleaved or nicked by proteases to become neuroactive. The bacterial strains that make botulinum toxin serotypes A and G possess endogenous proteases and serotypes A and G can therefore be recovered from bacterial cultures in predominantly their active form. In contrast, botulinum toxin serotypes C.sub.1, D and E are synthesized by nonproteolytic strains and are therefore typically unactivated when recovered from culture. Serotypes B and F are produced by both proteolytic and nonproteolytic strains and therefore can be recovered in either the active or inactive form. However, even the proteolytic strains that produce, for example, the botulinum toxin type B serotype only cleave a portion of the toxin produced. The exact proportion of nicked to unnicked molecules depends on the length of incubation and the temperature of the culture. Therefore, a certain percentage of any preparation of, for example, the botulinum toxin type B toxin is likely to be inactive, possibly accounting for the known significantly lower potency of botulinum toxin type B as compared to botulinum toxin type A. The presence of inactive botulinum toxin molecules in a clinical preparation will contribute to the overall protein load of the preparation, which has been linked to increased antigenicity, without contributing to its clinical efficacy. Additionally, it is known that botulinum toxin type B has, upon intramuscular injection, a shorter duration of activity and is also less potent than botulinum toxin type A at the same dose level.

Brief Summary Text (36):

High quality crystalline botulinum toxin type A can be produced from the Hall A strain of Clostridium botulinum with characteristics of .gtoreq.3.times.10.sup.7 U/mg, an A.sub.260 /A.sub.278 of less than 0.60 and a distinct pattern of banding

on gel electrophoresis. The known Shantz process can be used to obtain crystalline botulinum toxin type A, as set forth in Shantz, E. J., et al, Properties and Use of Botulinum Toxin and Other Microbial Neurotoxins in Medicine, Microbiol Rev. 56;80-99:1992. Generally, the botulinum toxin type A complex can be isolated and purified from an anaerobic fermentation by cultivating Clostridium botulinum type A in a suitable medium. The known process can also be used, upon separation out of the non-toxin proteins, to obtain pure botulinum toxins, such as for example: purified botulinum toxin type A with an approximately 150 kD molecular weight with a specific potency of 1-2.times.10.sup.8 LD.sub.50 U/mg or greater; purified botulinum toxin type B with an approximately 156 kD molecular weight with a specific potency of 1-2.times.10.sup.8 LD.sub.50 U/mg or greater, and; purified botulinum toxin type F with an approximately 155 kD molecular weight with a specific potency of 1-2.times.10.sup.7 LD.sub.50 U/mg or greater.

Brief Summary Text (37):

Botulinum toxins and/or botulinum toxin complexes can be obtained from various sources, including List Biological Laboratories, Inc., Campbell, Calif.; the Centre for Applied Microbiology and Research, Porton Down, U.K.; Wako (Osaka, Japan), Metabiologics (Madison, Wis.) as well as from Sigma Chemicals of St. Louis, Mo.

Brief Summary Text (38):

Pure botulinum toxin is so labile that it is generally not used to prepare a pharmaceutical composition. Furthermore, the botulinum toxin complexes, such as the toxin type A complex are also extremely susceptible to denaturation due to surface denaturation, heat, and alkaline conditions. Inactivated toxin forms toxoid proteins which can be immunogenic. The resulting antibodies can render a patient refractory to toxin injection.

Brief Summary Text (39):

As with enzymes generally, the biological activities of the botulinum toxins (which are intracellular peptidases) are dependent, at least in part, upon their three dimensional conformation. Thus, botulinum toxin type A is detoxified by heat, various chemicals surface stretching and surface drying. Additionally, it is known that dilution of the toxin complex obtained by the known culturing, fermentation and purification to the much, much lower toxin concentrations used for pharmaceutical composition formulation results in rapid detoxification of the toxin unless a suitable stabilizing agent is present. Dilution of the toxin from milligram quantities to a solution containing nanograms per milliliter presents significant difficulties because of the rapid loss of specific toxicity upon such great dilution. Additionally, the toxin may be used months or years after the toxin containing pharmaceutical composition is formulated. Significantly, it is known that the toxin can be stabilized during the manufacture and compounding processes as well as during storage by use of a stabilizing agent such as albumin and gelatin.

Brief Summary Text (40):

The commercially available botulinum toxin sold under the trademark BOTOX.RTM. (available from Allergan, Inc., of Irvine, Calif.). BOTOX.RTM. consists of a freeze-dried, purified botulinum toxin type A complex, albumin and sodium chloride packaged in sterile, vacuum-dried form. The botulinum toxin type A is made from a culture of the Hall strain of Clostridium botulinum grown in a medium containing N-Z Z amine and yeast extract. The botulinum toxin type A complex is purified from the culture solution by a series of acid precipitations to a crystalline complex consisting of the active high molecular weight toxin protein and an associated hemagglutinin protein. The crystalline complex is re-dissolved in a solution containing saline and albumin and sterile filtered (0.2 microns) prior to vacuum-drying. The vacuum-dried product is stored in a freezer at or below -5.degree. C. BOTOX.RTM. can be reconstituted with sterile, non-preserved saline prior to intramuscular injection. Each vial of BOTOX.RTM. contains about 100 units (U) of Clostridium botulinum toxin type A purified neurotoxin complex, 0.5 milligrams of

human serum albumin and 0.9 milligrams of sodium chloride in a sterile, vacuum-dried dried form without a preservative.

Brief Summary Text (42):

It has been reported that botulinum toxin type A has been used in various clinical settings, including the following: (1) about 75-125 units of BOTOX.RTM. per intramuscular injection (multiple muscles) to treat cervical dystonia; (2) 5-10 units of BOTOX.RTM. per intramuscular injection to treat glabellar lines (brow furrows) (5 units injected intramuscularly into the procerus muscle and 10 units injected intramuscularly into each corrugator superciliaris muscle); (3) about 30-80 units of BOTOX.RTM. to treat constipation by intrasphincter injection of the puborectalis muscle; (4) about 1-5 units per muscle of intramuscularly injected BOTOX.RTM. to treat blepharospasm by injecting the lateral pre-tarsal orbicularis oculi muscle of the upper lid and the lateral pre-tarsal orbicularis oculi of the lower lid. (5) to treat strabismus, extraocular muscles have been injected intramuscularly with between about 1-5 units of BOTOX.RTM., the amount injected varying based upon both the size of the muscle to be injected and the extent of muscle paralysis desired (i.e. amount of diopter correction desired). (6) to treat upper limb spasticity following stroke by intramuscular injections of BOTOX.RTM. into five different upper limb flexor muscles, as follows: (a) flexor digitorum profundus: 7.5 U to 30 U (b) flexor digitorum sublimus: 7.5 U to 30 U (c) flexor carpi ulnaris: 10 U to 40 U (d) flexor carpi radialis: 15 U to 60 U (e) biceps brachii: 50 U to 200 U. Each of the five indicated muscles has been injected at the same treatment session, so that the patient receives from 90 U to 360 U of upper limb flexor muscle BOTOX.RTM. by intramuscular injection at each treatment session. (7) to treat migraine, pericranial injected (injected symmetrically into glabellar, frontalis and temporalis muscles) injection of 25 U of BOTOX.RTM. has showed significant benefit as a prophylactic treatment of migraine compared to vehicle as measured by decreased measures of migraine frequency, maximal severity, associated vomiting and acute medication use over the three month period following the 25 U injection.

Brief Summary Text (43):

It is known that botulinum toxin type A can have an efficacy for up to 12 months (European J. Neurology 6 (Supp 4): S111-S1150:1999), and in some circumstances for as long as 27 months, (The Laryngoscope 109: 1344-1346:1999). However, the usual duration of the paralytic effect of an intramuscular injection of Botox.TM. is typically about 3 to 4 months.

Brief Summary Text (44):

The success of botulinum toxin type A to treat a variety of clinical conditions has led to interest in other botulinum toxin serotypes. A study of two commercially available botulinum type A preparations (BOTOX.RTM. and Dysport.RTM.) and preparations of botulinum toxins type B and F (both obtained from Wako Chemicals, Japan) has been carried out to determine local muscle weakening efficacy, safety and antigenic potential. Botulinum toxin preparations were injected into the head of of the right gastrocnemius muscle (0.5 to 200.0 units/kg) and muscle weakness was assessed using the mouse digit abduction scoring assay (DAS). ED.sub.50 values were calculated from dose response curves. Additional mice were given intramuscular injections to determine LD.sub.50 doses. The therapeutic index was calculated as LD.sub.50 /ED.sub.50. Separate groups of mice received hind limb injections of BOTOX.RTM. (5.0 to 10.0 units/kg) or botulinum toxin type B (50.0 to 400.0 units/kg), and were tested for muscle weakness and increased water consumption, the later being a putative model for dry mouth. Antigenic potential was assessed by monthly intramuscular injections in rabbits (1.5 or 6.5 ng/kg for botulinum toxin type B or 0.15 ng/kg for BOTOX.RTM.). Peak muscle weakness and duration were dose related for all serotypes. DAS ED.sub.50 values (units/kg) were as follows: BOTOX.RTM. : 6.7, Dysporte: 24.7, botulinum toxin type B: 27.0 to 244.0, botulinum toxin type F: 4.3. BOTOX.RTM. had a longer duration of action than botulinum toxin type B or botulinum toxin type F. Therapeutic index values were as follows:

BOTOX.RTM. : 10.5, Dysport.RTM.: 6.3, botulinum toxin type B: 3.2. Water consumption was greater in mice injected with botulinum toxin type B than with BOTOX.RTM., although botulinum toxin type B was less effective at weakening muscles. After four months of injections 2 of 4 (where treated with 1.5 ng/kg) and 4 of 4 (where treated with 6.5 ng/kg) rabbits developed antibodies against botulinum toxin type B. In a separate study, 0 of 9 BOTOX.RTM. treated rabbits demonstrated antibodies against botulinum toxin type A. DAS results indicate relative peak potencies of botulinum toxin type A being equal to botulinum toxin type F, and botulinum toxin type F being greater than botulinum toxin type B. With regard to duration of effect, botulinum toxin type A was greater than botulinum toxin type B, and botulinum toxin type B duration of effect was greater than botulinum toxin type F. As shown by the therapeutic index values, the two commercial preparations of botulinum toxin type A (BOTOX.RTM. and Dysport.RTM.) are different. The increased water consumption behavior observed following hind limb injection of botulinum toxin type B indicates that clinically significant amounts of of this serotype entered the murine systemic circulation. The results also indicate that in order to achieve efficacy comparable to botulinum toxin type A, it is necessary to increase doses of the other serotypes examined. Increased dosage can comprise safety. Furthermore, in rabbits, type B was more antigenic than was BOTOX.RTM., possibly because of the higher protein load injected to achieve an effective dose of botulinum toxin type B. Eur J Neurol 1999 Nov;6(Suppl 4):S3-S10.

Brief Summary Text (45):

In addition to having pharmacologic actions at a peripheral location, a botulinum toxin can also exhibit a denervation effect in the central nervous system. Wiegand et al, Naunyn-Schmiedeberg's Arch. Pharmacol. 1976;292, 161-165, and Habermann, Naunyn-Schmiedeberg's Arch. Pharmacol. 1974;281, 47-56 reported that botulinum toxin is able to ascend to the spinal area by retrograde transport. As such, a botulinum toxin injected at a peripheral location, for example intramuscularly, can potentially be retrograde transported to the spinal cord.

Brief Summary Text (46):

U.S. Pat. No. 5,989,545 discloses that a modified clostridial neurotoxin or fragment thereof, preferably a botulinum toxin, chemically conjugated or ,recombinantly fused to a particular targeting moiety can be used to treat pain by administration of the agent to the spinal cord.

Brief Summary Text (47):

At the present time, essentially all therapeutic use of a botulinum toxin is by subcutaneous or intramuscular injection of an aqueous solution of a botulinum toxin type A or B. Typically, a repeat injection must be administered every 2-4 months in order to maintain the therapeutic efficacy of the toxin (i.e. a reduction of muscle spasm at or in the vicinity of the injection site). Each administration of a dose of a botulinum toxin to a patient therefore requires the patient to present himself to his physician at regular intervals. Unfortunately, patients can forget or be unable to attend appointments and physician schedules can make regular, periodic care over a multiyear period difficult to consistently maintain. Additionally, the requirement for 3-6 toxin injections per year on an ongoing basis increases the risk of infection or of misdosing the patient.

Brief Summary Text (52):

Acetylcholine is released from cholinergic neurons when small, clear, intracellular vesicles fuse with the presynaptic neuronal cell membrane. A wide variety of non-neuronal secretory cells, such as, adrenal medulla (as well as the PC12 cell line) and pancreatic islet cells release catecholamines and parathyroid hormone, respectively, from large dense-core vesicles. The PC12 cell line is a clone of rat pheochromocytoma cells extensively used as a tissue culture model for studies of sympathoadrenal development. Botulinum toxin inhibits the release of both types of compounds from both types of cells in vitro, permeabilized (as by electroporation) or by direct injection of the toxin into the denervated cell. Botulinum toxin is

also known to block release of the neurotransmitter glutamate from cortical synaptosomes cell cultures.

Brief Summary Text (55):

The tetanus toxin bears many similarities to the botulinum toxins. Thus, both the tetanus toxin and the botulinum toxins are polypeptides made by closely related species of Clostridium (Clostridium tetani and Clostridium botulinum, respectively). Additionally, both the tetanus toxin and the botulinum toxins are dichain proteins composed of a light chain (molecular weight about 50 kD) covalently bound by a single disulfide bond to a heavy chain (molecular weight about 100 kD). Hence, the molecular weight of tetanus toxin and of each of the seven botulinum toxins (non-complexed) is about 150 kD. Furthermore, for both the tetanus toxin and the botulinum toxins, the light chain bears the domain which exhibits intracellular biological (protease) activity, while the heavy chain comprises the receptor binding (immunogenic) and cell membrane translocational domains.

Brief Summary Text (56):

Further, both the tetanus toxin and the botulinum toxins exhibit a high, specific affinity for gangliocide receptors on the surface of presynaptic cholinergic neurons. Receptor mediated endocytosis of tetanus toxin by peripheral cholinergic neurons results in retrograde axonal transport, blocking of the release of inhibitory neurotransmitters from central synapses and a spastic paralysis. Receptor mediated endocytosis of botulinum toxin by peripheral cholinergic neurons results in little if any retrograde transport, inhibition of acetylcholine exocytosis from the intoxicated peripheral motor neurons and a flaccid paralysis.

Brief Summary Text (57):

Finally, the tetanus toxin and the botulinum toxins resemble each other in both biosynthesis and molecular architecture. Thus, there is an overall 34% identity between the protein sequences of tetanus toxin and botulinum toxin type A, and a sequence identity as high as 62% for some functional domains. Binz T. et al., The Complete Sequence of Botulinum Neurotoxin Type A and Comparison with Other Clostridial Neurotoxins, J Biological Chemistry 265(16);9153-9158:1 990.

Brief Summary Text (58):

A toxoid is an antigen which can be used to raise antibodies to and thereby vaccinate against the toxin from which the toxoid is derived. Typically, the toxoid comprises the immunogenic fragment of the toxin (i.e. the carboxyl terminal of the heavy chain (designed as H.sub.c) of the tetanus toxin or the botulinum toxins) or a toxin rendered biologically inactive, though still immunogenic, by thermal or chemical (i.e. formalin treatment) denaturation or alteration of the native toxin. Thus, unlike the natural toxin, the toxoid derived from the tetanus or botulinum toxin has been derived of its biological activity, that is its ability to act as an intracellular protease and inhibit neuronal exocytosis of acetylcholine.

Brief Summary Text (65):

Tetanus and botulinum toxoid vaccines have been made by treating the native toxin with formalin. The U.S. Center for Disease Control can supply a pentavalent, formalin-inactivated toxoid of botulinum toxin types A, B, C, D and E. The pre-exposure immunization schedule calls for subcutaneous administration of the botulinum toxoid vaccine in three dosings at 0, 2 and 12 weeks with a booster at plus 12 months and yearly boosters at yearly intervals thereafter if antibody levels fall.

Brief Summary Text (66):

U.S. Pat. No. 5,980,948 discusses use of polyetherester copolymer microspheres for encapsulation and controlled delivery of a variety of protein drugs, including tetanus and botulinum antitoxins.

Brief Summary Text (67):

U.S. Pat. No. 5,902,565 discusses A controlled or delayed-release preparation comprising microspherical particles comprising a continuous matrix of biodegradable polymer containing discrete, immunogen-containing regions, where the immunogens can be botulinum toxin type C and D toxoids.

Brief Summary Text (68):

What is needed therefore is a biocompatible, pulsatile release, botulinum toxin delivery system by which therapeutic amounts of the botulinum toxin can be locally administered in vivo to a human patient over a prolonged period of time.

Brief Summary Text (70):

The present invention meets this need and provides a biocompatible, pulsatile release, botulinum toxin delivery system by which therapeutic amounts of the botulinum toxin can be locally administered in vivo to a human patient over a prolonged period of time.

Brief Summary Text (71):

The present invention provides a botulinum toxin implant which overcomes the known problems, difficulties and deficiencies associated with repetitive bolus or subcutaneous injection of a botulinum toxin, to treat an affliction such as a movement disorder, including a muscle spasm.

Brief Summary Text (72):

A pulsatile release botulinum toxin delivery system within the scope of the present invention can comprise a carrier material and a botulinum toxin associated with the carrier. The toxin can be associated with the carrier by being mixed with and encapsulated by the carrier to thereby form a pulsatile release botulinum toxin delivery system, that is a botulinum toxin implant. The implant can release therapeutic amounts of the botulinum toxin from the carrier in a plurality of pulses in vivo upon subdermal implantation of the implant system into a human patient. "Subdermal" implantation includes subcutaneous, intramuscular, intraglandular and intracranial sites of implantation.

Brief Summary Text (73):

Preferably, the carrier comprises a plurality of polymeric microspheres (i.e. a polymeric matrix) and substantial amounts of the botulinum toxin has not be transformed into a botulinum toxoid prior to association of the botulinum toxin with the carrier. That is, significant amounts of the botulinum toxin associated with the carrier have a toxicity which is substantially unchanged relative to the toxicity of the botulinum toxin prior to association of the botulinum toxin with the carrier.

Brief Summary Text (74):

According to the present invention, the botulinum toxin can be released from the carrier over of a period of time of from about 10 days to about 6 years and the carrier is comprised of a substance which is substantially biodegradable. The botulinum toxin is one of the botulinum toxin types A, B, C.sub.1, D, E, F and G and and is preferably botulinum toxin type A. The botulinum toxin can be associated with the carrier in an amount of between about 1 unit and about 50,000 units of the botulinum toxin. Preferably, the quantity of the botulinum toxin associated with the carrier is between about 10 units and about 2,000 units of a botulinum toxin type A. Where the botulinum toxin is botulinum toxin type B, preferably, the quantity of the botulinum toxin associated with the carrier is between about 100 units and about 30,000 units of a botulinum toxin type B.

Brief Summary Text (75):

A detailed embodiment of the present invention can comprise a controlled release system, comprising a biodegradable polymer and between about 10 units and about 100,000 units of a botulinum toxin encapsulated by the polymer carrier, thereby

forming a controlled release system, wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a pulsatile manner in vivo upon subdermal implantation of the controlled release system in a human patient over a prolonged period of time extending from about 2 months to about 5 years.

Brief Summary Text (76):

A method for making an implant within the scope of the present invention can have the steps of: dissolving a polymer in a solvent to form a polymer solution; mixing or dispersing a botulinum toxin in the polymer solution to form a polymerbotulinum toxin mixture, and; allowing the polymer-botulinum toxin mixture to set or cure, thereby making an implant for pulsatile release of the botulinum toxin. This method can have the further step after the mixing step of evaporating solvent.

Brief Summary Text (77):

A method for using a pulsatile implant within the scope of the present invention can be by injecting or implanting a polymeric implant which includes a botulinum toxin, thereby treating a movement disorder or a disorder influenced by cholinergic innervation by local administration of a botulinum toxin.

Brief Summary Text (78):

An alternate embodiment of the present invention can be a carrier comprising a polymer selected from the group of polymers consisting of polylactides and polyglycolides and a stabilized botulinum toxin associated with the carrier, thereby forming a pulsatile release botulinum toxin delivery system, wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a plurality of pulses in vivo upon subdermal implantation of the delivery system in a human patient. The carrier can comprise a plurality of discrete sets of polymeric, botulinum toxin incorporating microspheres, wherein each set of polymers has a different polymeric composition.

Brief Summary Text (79):

The botulinum toxin used in an implant according to the present invention can comprise: a first element comprising a binding element able to specifically bind to a neuronal cell surface receptor under physiological conditions, a second element comprising a translocation element able to facilitate the transfer of a polypeptide across a neuronal cell membrane, and a third element comprising a therapeutic element able, when present in the cytoplasm of a neuron, to inhibit exocytosis of acetylcholine from the neuron. The therapeutic element can cleave a SNARE protein, thereby inhibiting the exocytosis of acetylcholine from the neuron and the SNARE protein is can be selected from the group consisting of syntaxin, SNAP-25 and VAMP. Generally, the neuron affected by the botulinum toxin is a presynaptic, cholinergic, cholinergic, peripheral motor neuron.

Brief Summary Text (80):

The amount of a botulinum toxin administered by a continuous release system within the scope of the present invention during a given period can be between about 10.sup.-3 U/kg and about 35 U/kg for a botulinum toxin type A and up to about 2000 U/kg for other botulinum toxins, such as a botulinum toxin type B. 35 U/kg or 2000 U/kg is an upper limit because it approaches a lethal dose of certain neurotoxins, such as botulinum toxin type A or botulinum toxin type B, respectively. Thus, it has been reported that about 2000 units/kg of a commercially available botulinum toxin type B preparation approaches a primate lethal dose of type B botulinum toxin. Meyer K. E. et al, A Comparative Systemic Toxicity Study of Neurobloc in Adult Juvenile Cynomolgus Monkeys, Mov. Disord 15(Suppl 2);54;2000.

Brief Summary Text (81):

Preferably, the amount of a type A botulinum toxin administered by a continuous release system during a given period is between about 10.sup.-2 U/kg and about 25 U/kg. Preferably, the amount of a type B botulinum toxin administered by a continuous release system during a given period is between about 10.sup.-2 U/kg and

and about 1000 U/kg, since it has been reported that less than about 1000 U/kg of type B botulinum toxin can be intramuscularly administered to a primate without systemic effect. Ibid. More preferably, the type A botulinum toxin is administered in an amount of between about 10 sup.-1 U/kg and about 15 U/kg. Most preferably, the type A botulinum toxin is administered in an amount of between about 1 U/kg and about 10 U/kg. In many instances, an administration of from about 1 units to about 500 units of a botulinum toxin type A, provides effective and long lasting therapeutic relief. More preferably, from about 5 units to about 300 units of a botulinum toxin, such as a botulinum toxin type A, can be used and most preferably, from about 10 units to about 200 units of a neurotoxin, such as a botulinum toxin type A, can be locally administered into a target tissue with efficacious results. In a particularly preferred embodiment of the present invention from about 1 units to about 100 units of a botulinum toxin, such as botulinum toxin type A, can be locally administered into a target tissue with therapeutically effective results.

Brief Summary Text (82):

The botulinum toxin can be made by Clostridium botulinum. Additionally, the botulinum toxin can be a modified botulinum toxin, that is a botulinum toxin that has at least one of its amino acids deleted, modified or replaced, as compared to the native or wild type botulinum toxin. Furthermore, the botulinum toxin can be a recombinant produced botulinum toxin or a derivative or fragment thereof.

Brief Summary Text (83):

Significantly, the botulinum toxin can be administered by subdermal implantation to the patient by placement of a botulinum toxin implant. The botulinum toxin can be administered to a muscle of a patient in an amount of between about 1 unit and about 10,000 units. When the botulinum toxin is botulinum toxin type A and the botulinum toxin can be administered to a muscle of the patient in an amount of between about 1 unit and about 100 units.

Brief Summary Text (84):

Notably, it has been reported that glandular tissue treated by a botulinum toxin can show a reduced secretory activity for as long as 27 months post injection of the toxin. Laryngoscope 1999;109:1344-1346, Laryngoscope 1998;108:381-384.

Brief Summary Text (85):

The present invention relates to an implant for the controlled release of a neurotoxin and to methods for making and using such implants. The implant can comprise a polymer matrix containing a botulinum toxin. The implant is designed to administer effective levels of neurotoxin over a prolonged period of time when administered, for example, intramuscularly, epidurally or subcutaneously for the treatment of various diseases conditions.

Brief Summary Text (86):

This invention further relates to a composition, and methods of making and using the composition, for the controlled release of biologically active, stabilized neurotoxin. The controlled release composition of this invention can comprise a polymeric matrix of a biocompatible polymer and biologically active, stabilized neurotoxin dispersed within the biocompatible polymer.

Brief Summary Text (91):

"Biologically active compound" means a compound which can effect a beneficial change in the subject to which it is administered. For example, "biologically active compounds" include neurotoxins.

Brief Summary Text (97):

"Neurotoxin" means an agent which can interrupt nerve impulse transmission across a neuromuscular or neuroglandular junction, block or reduce neuronal exocytosis of a neurotransmitter or alter the action potential at a sodium channel voltage gate of a neuron. Examples of neurotoxins include botulinum toxins, tetanus toxins,

saxitoxins, and tetrodotoxin.

Brief Summary Text (99):

A method for making an implant within the scope of the present invention for controlled release of a neurotoxin, can include dissolving a biocompatible polymer in a polymer solvent to form a polymer solution, dispersing particles of biologically active, stabilized neurotoxin in the polymer solution, and then solidifying the polymer to form a polymeric matrix containing a dispersion of the neurotoxin particles.

Brief Summary Text (100):

A method of using an implant within the scope of the present invention forming for controlled release of a neurotoxin can comprise providing a therapeutically effective level of biologically active, neurotoxin in a patient for a prolonged period of time by implanting in the patient the implant.

Brief Summary Text (102):

The present invention is based upon the discovery of a pulsatile release implant comprising a biocompatible, biodegradable polymer capable of exhibiting in vivo multiphasic release of therapeutic amounts of a botulinum toxin over a prolonged period of time.

Brief Summary Text (103):

A botulinum toxin delivery system within the scope of the present invention is capable of pulsatile (i.e. multiphasic) release of therapeutic amounts of a botulinum toxin. By pulsatile release it is meant that during a period of time, which can extend from about 1 hour to about 4 weeks, a quantity of therapeutically effective (i.e. biologically active) botulinum toxin is released from a carrier material in vivo at the site of implantation. The pulse of released botulinum toxin can comprise (for a botulinum toxin type A) as little as about 1 unit (i.e. to treat treat blepharospasm) to as much as 200 units (i.e. to treat of a large spasmodic muscle, such as the biceps). The quantity of botulinum toxin required for therapeutic efficacy can be varied according to the known clinical potency of the different botulinum toxin serotypes. For example, several orders of magnitude more units of a botulinum toxin type B are typically required to achieve a physiological effect comparable to that achieved from use of a botulinum toxin type A. Prior to and following each pulse there is a period of reduced or substantially no botulinum toxin release from the implant.

Brief Summary Text (104):

The botulinum toxin released in therapeutically effective amounts by a controlled release delivery system within the scope of the present invention is preferably, substantially biologically active botulinum toxin. In other words, the botulinum toxin released from the disclosed delivery system is capable of binding with high affinity to a cholinergic neuron, being translocated, at least in part, across the neuronal membrane, and through its activity in the cytosol of the neuron of inhibiting exocytosis of acetylcholine from the neuron. The present invention excludes from its scope use deliberate use of a botulinum toxoid as an antigen in order to confer immunity to the botulinum toxin through development of antibodies (immune response) due to the immunogenicity of the toxoid. The purpose of the present invention is to permit a controlled release of minute amounts of a botulinum toxin from a delivery system so as to inhibit exocytosis in vivo and thereby achieve a desired therapeutic effect, such as reduction of muscle spasm or muscle tone, preventing a muscle from contracting or to reduce an excessive secretion (i.e. a sweat secretion) from a cholinergically influenced secretory cell or gland.

Brief Summary Text (105):

Pulsatile release of a botulinum toxin from an implant can be accomplished by preparing a plurality of implants with differing carrier material compositions. For

example, holding other factors, such as polymer molecular weight, constant an implant can be made up of a several sets of botulinum toxin encapsulated microspheres, each set of microspheres having a different polymer composition such that the polymers of each set of microspheres degrade, and release toxin, at differing rates. Conveniently, the plurality of sets of differing polymer composition microspheres can be pressed into the form of a disc, and implanted as a pellet. The pulsatile release implant can be implanted subcutaneously, intramuscularly, intracranially, intraglandular, etc, at a site so that systemic entry of the toxin is not encouraged.

Brief Summary Text (106):

A first pulse of a botulinum toxin can be locally administered due to the presence of a botulinum toxin (i.e. free or non-implant incorporated botulinum toxin) administered in conjunction with and at the same time as insertion of the implant and/or due to a burst effect of botulinum toxin release from the implanted microspheres. A second pulse of a botulinum toxin can be administered by the implant at about three months post implantation upon biodegradation of a first set of microspheres. A third pulse of a botulinum toxin can be delivered by the system at about six months post implantation upon dissolution of a second set of bioerodible microspheres, and so on. Thus, a botulinum toxin delivery system within the scope of the present invention which comprises three differing sets of appropriate microsphere polymer compositions, permits a patient to be reimplant or reinvested with a botulinum toxin only once every 12 months.

Brief Summary Text (107):

For example, it is known that biodegradable PLA:PGA microspheres can be made with varying copolymer content such that proportionally different polymer degradation time windows result. Thus, a 75:25 lactide:glycolide polymer can degrade at about ninety days post implantation. Additionally, a 100:0 lactide:glycolide polymer can degrade at about one hundred and eighty days post implantation. Furthermore, a 95:5 poly(DL-lactide):glycolide polymer can degrade at about two hundred and seventy days post implantation. Finally, a 100:0 poly(DL-lactide):glycolide polymer can degrade at about twelve months post implantation. See e.g. Kissel et al, Microencapsulation of Antigens Using Biodegradable Polymers: Facts and Fantasies, Behring Inst. Mitt., 98;1 72-183:1997; Cleland J. L., et al, Development of a Single-Shot Subunit Vaccine for HOV-1: Part 4. Optimizing Microencapsulation and Pulsatile Release of MN rpg120 from Biodegradable Microspheres, J Cont Rel 47;135-150:1997, and; Lewis D. H., Controlled Release of Bioactive Agents from Lactide/Glycolide Polymers, pages 1-41 of Chasin M., et al, "Biodegradable Polymers as Drug-Delivery Systems", Marcel Dekker, N.Y. (1990). The above-specified four discrete sets of polymeric microspheres can be prepared as botulinum toxin incorporating microspheres, and combined into a single implant capable of pulsatile release of the botulinum toxin over a one year period, thereby providing a patient treatment period per implant of about 15-16 months.

Brief Summary Text (108):

The delivery system is prepared so that the botulinum toxin is substantially uniformly dispersed in a biodegradable carrier. An alternate pulsatile delivery system within the scope of the present invention can comprise a carrier coated by a biodegradable coating, either the thickness of the coating or the coating material being varied, such that in the different sets of microspheres, the respective coating take from 3, 6, 9, etc months to be dissolved, thereby providing the desired toxin pulses. The microspheres are inert and are of such a size or due to being pressed into a disc, that they do not diffuse significantly beyond the site of injection. Hence, multiple implantations, as by needle injection, can be carried out at the same time.

Brief Summary Text (109):

A third embodiment within the scope of the present invention of a pulsatile, implant can comprise a non-porous, non-biodegradable, biocompatible tube which is

closed at one end. Carrier associated neurotoxin is interspaced discrete locations within the bore of the tube. Thus, toxin at an open or porous, or erodible plug sealed pug the end of the tube rapidly diffuses out, causing the first local administration. Toxin further from the end of the tube takes longer to diffuse out and results in the second local

Brief Summary Text (110):

The thickness of the implant can be used to control the absorption of water by, and thus the rate of release of a neurotoxin from, a composition of the invention, thicker implants releasing the polypeptide more slowly than thinner ones.

Brief Summary Text (111):

The neurotoxin in a neurotoxin controlled release composition can also be mixed with with other excipients, such as bulking agents or additional stabilizing agents, such as buffers to stabilize the neurotoxin during lyophilization.

Brief Summary Text (114):

Biodegradable PLGA polymers have been used to form resorbable sutures and bone plates and in several commercial microparticle formulations. PLGA degrades through bulk erosion to produce lactic and glycolic acid and is commercially available in a variety of molecular weight and polymer end groups (e.g. lauryl alcohol or free acid). Polyanhydrides are another group of polymers that have been approved for use I humans, and have been used to deliver proteins and antigens. Unlike PLGA, polyanhydrides degrade by surface erosion, releasing neurotoxin entrapped at the carrier surface.

Brief Summary Text (115):

To prepare a suitable implant, the carrier polymer is dissolved in an organic solvent such as methylene chloride or ethyl acetate and the botulinum toxin is then mixed into the polymer solution. The conventional processes for microsphere formation are solvent evaporation and solvent (coacervation) methods. The water-in-oil-in-water (W/O/W) double emulsion method is a widely used method of protein antigen encapsulation into PLGA microspheres.

Brief Summary Text (116):

An aqueous solution of a botulinum toxin can be used to make a pulsatile implant. An aqueous solution of the neurotoxin is added to the polymer solution (polymer previously dissolved in a suitable organic solvent). The volume of the aqueous (neurotoxin) solution relative to the volume of organic (polymer) solvent is an important parameter in the determination of both the release characteristics of the microspheres and with regard to the encapsulation efficiency (ratio of theoretical to experimental protein loading) of the neurotoxin.

Brief Summary Text (118):

Thus, with a low aqueous phase (neurotoxin) to organic phase (polymer) volume ratio (i.e. aqueous volume:organic volume is .1toeq.0.1 ml/ml) essentially 100% of the neurotoxin can be encapsulated by the microspheres and the microspheres can show a triphasic release: an initial burst (first pulse), a lag phase with little or no neurotoxin being released and a second release phase (second pulse).

Brief Summary Text (119):

The length of the lag phase is dependent upon the polymer degradation rate which is in turn dependant upon polymer composition and molecular weight. Thus, the lag phase between the first (burst) pulse and the second pulse increases as the lactide content is increased, or as the polymer molecular weight is increased with the lactide:glycolide ratio being held constant. In addition to a low aqueous phase (neurotoxin) volume, operation at low temperature (2-8 degrees C.), as set forth above, increases the encapsulation efficiency, as well as reducing the initial burst and promoting increased neurotoxin stability against thermal inactivation

Brief Summary Text (120):

Suitable implants within the scope of the present invention for the controlled in vivo release of a neurotoxin, such as a botulinum toxin, can be prepared so that the implant releases the neurotoxin in a pulsatile manner. A pulsatile release implant can release a neurotoxin in a biphasic or multiphase manner. Thus, a pulsatile release implant can have a relatively short initial induction (burst) period, followed by periods during which reduced, little or no neurotoxin is released.

Brief Summary Text (121):

A controlled release of biologically active neurotoxin is a release which results in therapeutically effective, with negligible serum levels, of biologically active, neurotoxin over a period longer than that obtained following direct administration of aqueous neurotoxin. It is preferred that a controlled release be a release of neurotoxin for a period of about six months or more, and more preferably for a period of about one year or more.

Brief Summary Text (123):

Denaturation of the encapsulated neurotoxin in the body at 37 degrees C. for a prolonged period of time can be reduced by stabilizing the neurotoxin by lyophilizing it with albumin, lyophilizing from an acidic solution, lyophilizing from a low moisture content solution (these three criteria can be met with regard to a botulinum toxin type A by use of non-reconstituted Botox.RTM.) and using a specific polymer matrix composition.

Brief Summary Text (124):

Preferably, the release of biologically active neurotoxin in vivo does not result in a significant immune system response during the release period of the neurotoxin.

Brief Summary Text (125):

A pulsatile botulinum toxin delivery system preferably permits botulinum release from biodegradable polymer microspheres in a biologically active form, that is with a substantially native toxin conformation. To stabilize a neurotoxin, both in a format which renders the neurotoxin useful for mixing with a suitable polymer which can form the implant matrix (i.e. a powdered neurotoxin which has been freeze dried or lyophilized) as well as while the neurotoxin is present or incorporated into the matrix of the selected polymer, various pharmaceutical excipients can be used. Suitable excipients can include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, albumin and dried skim milk. The neurotoxin in a neurotoxin controlled release composition can be mixed with excipients, bulking bulking agents and stabilizing agents, and buffers to stabilize the neurotoxin during lyophilization or freeze drying.

Brief Summary Text (126):

It has been discovered that a stabilized neurotoxin can comprise biologically active, non-aggregated neurotoxin complexed with at least one type of multivalent metal cation which has a valiancy of +2 or more.

Brief Summary Text (128):

Preferably, the molar ratio of metal cation component to neurotoxin, for the metal cation stabilizing the neurotoxin, is between about 4:1 to about 100:1 and more typically about 4:1 to about 10:1.

Brief Summary Text (129):

A preferred metal cation used to stabilize a botulinum toxin is Zn.sup.++ because the botulinum toxin are known to be zinc endopeptidases. Divalent zinc cations are preferred because botulinum toxin is known to be a divalent zinc endopeptidase. In a more preferred embodiment, the molar ratio of metal cation component, containing

Zn.sup.++ cations, to neurotoxin is about 6:1.

Brief Summary Text (130):

The suitability of a metal cation for stabilizing neurotoxin can be determined by one of ordinary skill in the art by performing a variety of stability indicating techniques such as polyacrylamide gel electrophoresis, isoelectric focusing, reverse phase chromatography, HPLC and potency tests on neurotoxin lyophilized particles containing metal cations to determine the potency of the neurotoxin after lyophilization and for the duration of release from microparticles. In stabilized neurotoxin, the tendency of neurotoxin to aggregate within a microparticle during hydration in vivo and/or to lose biological activity or potency due to hydration or due to the process of forming a controlled release composition, or due to the chemical characteristics of a controlled release composition, is reduced by complexing at least one type of metal cation with neurotoxin prior to contacting the neurotoxin with a polymer solution.

Brief Summary Text (131):

By the present invention, stabilized neurotoxin is stabilized against significant aggregation in vivo over the controlled release period. Significant aggregation is defined as an amount of aggregation resulting in aggregation of about 15% or more of the polymer encapsulated or polymer matrix incorporated neurotoxin. Preferably, aggregation is maintained below about 5% of the neurotoxin. More preferably, aggregation is maintained below about 2% of the neurotoxin present in the polymer.

Brief Summary Text (132):

In another embodiment, a neurotoxin controlled release composition also contains a second metal cation component, which is not contained in the stabilized neurotoxin particles, and which is dispersed within the polymer. The second metal cation component preferably contains the same species of metal cation, as is contained in the stabilized neurotoxin. Alternately, the second metal cation component can contain one or more different species of metal cation.

Brief Summary Text (133):

The second metal cation component acts to modulate the release of the neurotoxin from the polymeric matrix of the controlled release composition, such as by acting as a reservoir of metal cations to further lengthen the period of time over which the neurotoxin is stabilized by a metal cation to enhance the stability of neurotoxin in the composition.

Brief Summary Text (134):

A metal cation component used in modulating release typically contains at least one type of multivalent metal cation. Examples of second metal cation components suitable to modulate neurotoxin release, include, or contain, for instance, Mg(OH).sub.2, MgCO.sub.3 (such as 4MgCO.sub.3 Mg(OH).sub.2 5H.sub.2 O), ZnCO.sub.3 (such as 3Zn(OH).sub.2 2ZnCO.sub.3), CaCO.sub.3, Zn.sub.3 (C.sub.6 H.sub.5 O.sub.7).sub.2, Mg(OAc).sub.2, MgSO.sub.4, Zn(OAc).sub.2, ZnSO.sub.4, ZnCl.sub.2, MgCl.sub.2 and Mg.sub.3 (C.sub.6 H.sub.5 O.sub.7).sub.2. A suitable ratio of second metal cation component-to-polymer is between about 1:99 to about 1:2 by weight. The optimum ratio depends upon the polymer and the second metal cation component utilized.

Brief Summary Text (135):

The neurotoxin controlled release composition of this invention can be formed into many shapes such as a film, a pellet, a cylinder, a disc or a microsphere. A microsphere, as defined herein, comprises a polymeric component having a diameter of less than about one millimeter and having stabilized neurotoxin dispersed therein. A microsphere can have a spherical, non-spherical or irregular shape. It is preferred that a microsphere be spherical in shape. Typically, the microsphere will be of a size suitable for injection. A preferred size range for microspheres is from about 1 to about 180 microns in diameter.

Brief Summary Text (136):

In the method of this invention for forming a composition for the controlled release of biologically active, non-aggregated neurotoxin, a suitable amount of particles of biologically active, stabilized neurotoxin are dispersed in a polymer solution.

Brief Summary Text (137):

A suitable polymer solvent, as defined herein, is solvent in which the polymer is soluble but in which the stabilized neurotoxin is are substantially insoluble and non-reactive. Examples of suitable polymer solvents include polar organic liquids, such as methylene chloride, chloroform, ethyl acetate and acetone.

Brief Summary Text (138):

To prepare biologically active, stabilized neurotoxin, neurotoxin is mixed in a suitable aqueous solvent with at least one suitable metal cation component under pH conditions suitable for forming a complex of metal cation and neurotoxin. Typically, the complexed neurotoxin will be in the form of a cloudy precipitate, which is suspended in the solvent. However, the complexed neurotoxin can also be in solution. In an even more preferred embodiment, neurotoxin is complexed with Zn.sup.++.

Brief Summary Text (139):

Suitable pH conditions to form a complex of neurotoxin typically include pH values between about 5.0 and about 6.9. Suitable pH conditions are typically achieved through use of an aqueous buffer, such as sodium bicarbonate, as the solvent.

Brief Summary Text (140):

Suitable solvents are those in which the neurotoxin and the metal cation component are each at least slightly soluble, such as in an aqueous sodium bicarbonate buffer. For aqueous solvents, it is preferred that water used be either deionized water or water-for-injection (WFI).

Brief Summary Text (141):

The neurotoxin can be in a solid or a dissolved state, prior to being contacted with with the metal cation component. Additionally, the metal cation component can be in a solid or a dissolved state, prior to being contacted with the neurotoxin. In a preferred embodiment, a buffered aqueous solution of neurotoxin is mixed with an aqueous solution of the metal cation component.

Brief Summary Text (142):

Typically, the complexed neurotoxin will be in the form of a cloudy precipitate, which is suspended in the solvent. However, the complexed neurotoxin can also be in solution. In a preferred embodiment, the neurotoxin is complexed with Zn.sup.++.

Brief Summary Text (143):

The Zn.sup.++ complexed neurotoxin can then be dried, such as by lyophilization, to form particulates of stabilized neurotoxin. The Zn.sup.++ complexed neurotoxin, which is suspended or in solution, can be bulk lyophilized or can be divided into smaller volumes which are then lyophilized. In a preferred embodiment, the Zn.sup.++ complexed neurotoxin suspension is micronized, such as by use of an ultrasonic nozzle, and then lyophilized to form stabilized neurotoxin particles. Acceptable means to lyophilize the Zn.sup.++ complexed neurotoxin mixture include those known in the art.

Brief Summary Text (144):

In another embodiment, a second metal cation component, which is not contained in the stabilized neurotoxin particles, is also dispersed within the polymer solution.

Brief Summary Text (145):

It is understood that a second metal cation component and stabilized neurotoxin can be dispersed into a polymer solution sequentially, in reverse order, intermittently, separately or through concurrent additions. Alternately, a polymer, a second metal cation component and stabilized neurotoxin and can be mixed into a polymer solvent sequentially, in reverse order, intermittently, separately or through concurrent additions. In this method, the polymer solvent is then solidified to form a polymeric matrix containing a dispersion of stabilized neurotoxins.

Brief Summary Text (146):

A suitable method for forming an neurotoxin controlled release composition from a polymer solution is the solvent evaporation method is described in U.S. Pat. Nos. 3,737,337; 3,523,906; 3,691,090, and; 4,389,330. Solvent evaporation can be used as a method to form neurotoxin controlled release microparticles.

Brief Summary Text (147):

In the solvent evaporation method, a polymer solution containing a stabilized neurotoxin particle dispersion, is mixed in or agitated with a continuous phase; in which the polymer solvent is partially miscible, to form an emulsion. The continuous phase is usually an aqueous solvent. Emulsifiers are often included in the continuous phase to stabilize the emulsion. The polymer solvent is then evaporated over a period of several hours or more, thereby solidifying the polymer to form a polymeric matrix having a dispersion of stabilized neurotoxin particles contained therein.

Brief Summary Text (148):

A preferred method for forming neurotoxin controlled release microspheres from a polymer solution is described in U.S. Pat. No. 5,019,400. This method of microsphere formation, as compared to other methods, such as phase separation, additionally reduces the amount of neurotoxin required to produce a controlled release composition with a specific neurotoxin content.

Brief Summary Text (149):

In this method, the polymer solution, containing the stabilized neurotoxin dispersion, is processed to create droplets, wherein at least a significant portion of the droplets contain polymer solution and the stabilized neurotoxin. These droplets are then frozen by means suitable to form microspheres. Examples of means for processing the polymer solution dispersion to form droplets include directing the dispersion through an ultrasonic nozzle, pressure nozzle, Rayleigh jet, or by other known means for creating droplets from a solution.

Brief Summary Text (150):

The solvent in the frozen microdroplets is extracted as a solid and/or liquid into the non-solvent to form stabilized neurotoxin containing microspheres. Mixing ethanol with other non-solvents, such as hexane or pentane, can increase the rate of solvent extraction, above that achieved by ethanol alone, from certain polymers, such as poly(lactide-co-glycolide) polymers.

Brief Summary Text (151):

Yet another method of forming a neurotoxin implant, from a polymer solution, includes film casting, such as in a mold, to form a film or a shape. For instance, after putting the polymer solution containing a dispersion of stabilized neurotoxin into a mold, the polymer solvent is then removed by means known in the art, or the temperature of the polymer solution is reduced, until a film or shape, with a consistent dry weight, is obtained.

Brief Summary Text (152):

In the case of a biodegradable polymer implant, release of neurotoxin due to degradation of the polymer. The rate of degradation can be controlled by changing

polymer properties that influence the rate of hydration of the polymer. These properties include, for instance, the ratio of different monomers, such as lactide and glycolide, comprising a polymer; the use of the L-isomer of a monomer instead of of a racemic mixture; and the molecular weight of the polymer. These properties can affect hydrophilicity and crystallinity, which control the rate of hydration of the polymer. Hydrophilic excipients such as salts, carbohydrates and surfactants can also be incorporated to increase hydration and which can alter the rate of erosion of the polymer.

Brief Summary Text (153):

By altering the properties of a biodegradable polymer, the contributions of diffusion and/or polymer degradation to neurotoxin release can be controlled. For example, increasing the glycolide content of a poly(lactide-co-glycolide) polymer and decreasing the molecular weight of the polymer can enhance the hydrolysis of the polymer and thus, provides an increased neurotoxin release from polymer erosion. In addition, the rate of polymer hydrolysis is increased in non-neutral pH's. Therefore, an acidic or a basic excipient can be added to the polymer solution, used to form the microsphere, to alter the polymer erosion rate.

Brief Summary Text (154):

An implant within the scope of the present invention can be administered to a human, or other animal, by any non-systemic means of administration, such as by implantation (e.g. subcutaneously, intramuscularly, intracranially, intravaginally and intradermally), to provide the desired dosage of neurotoxin based on the known parameters for treatment with neurotoxin of various medical conditions, as previously set forth.

Brief Summary Text (155):

The specific dosage by implant appropriate for administration is readily determined by one of ordinary skill in the art according to the factor discussed above. The dosage can also depend upon the size of the tissue mass to be treated or denervated, and the commercial preparation of the toxin. Additionally, the estimates for appropriate dosages in humans can be extrapolated from determinations of the amounts of botulinum required for effective denervation of other tissues. Thus, the amount of botulinum A to be injected is proportional to the mass and level of activity of the tissue to be treated. Generally, between about 0.01 units per kilogram to about 35 units per kg of patient weight of a botulinum toxin, such as botulinum toxin type A, can be released by the present implant per unit time period (i.e. over a period of or once every 2-4 months) to effectively accomplish a desired muscle paralysis. Less than about 0.01 U/kg of a botulinum toxin does not have a significant therapeutic effect upon a muscle, while more than about 35 U/kg of a botulinum toxin approaches a toxic dose of a neurotoxin, such as a botulinum toxin type A. Careful preparation and placement of the implant prevents significant amounts of a botulinum toxin from appearing systemically. A more preferred dose range is from about 0.01 U/kg to about 25 U/kg of a botulinum toxin, such as that formulated as BOTOX.RTM.. The actual amount of U/kg of a botulinum toxin to be administered depends upon factors such as the extent (mass) and level of activity of the tissue to be treated and the administration route chosen. Botulinum toxin type A is a preferred botulinum toxin serotype for use in the methods of the present present invention.

Brief Summary Text (156):

Preferably, a neurotoxin used to practice a method within the scope of the present invention is a botulinum toxin, such as one of the serotype A, B, C, D, E, F or G botulinum toxins. Preferably, the botulinum toxin used is botulinum toxin type A, because of its high potency in humans, ready availability, and known safe and efficacious use for the treatment of skeletal muscle and smooth muscle disorders when locally administered by intramuscular injection.

Brief Summary Text (157):

The present invention includes within its scope the use of any neurotoxin which has a long duration therapeutic effect when used to treat a movement disorder or an affliction influenced by cholinergic innervation. For example, neurotoxins made by any of the species of the toxin producing Clostridium bacteria, such as Clostridium botulinum, Clostridium butyricum, and Clostridium beratti can be used or adapted for use in the methods of the present invention. Additionally, all of the botulinum serotypes A, B, C, D, E, F and G can be advantageously used in the practice of the present invention, although type A is the most preferred serotype, as explained above. Practice of the present invention can provide effective relief for from 1 month to about 5 or 6 years.

Brief Summary Text (158):

The present invention includes within its scope: (a) neurotoxin complex as well as pure neurotoxin obtained or processed by bacterial culturing, toxin extraction, concentration, preservation, freeze drying and/or reconstitution and; (b) modified or recombinant neurotoxin, that is neurotoxin that has had one or more amino acids or amino acid sequences deliberately deleted, modified or replaced by known chemical/biochemical amino acid modification procedures or by use of known host cell/recombinant vector recombinant technologies, as well as derivatives or fragments of neurotoxins so made, and includes neurotoxins with one or more attached attached targeting moieties for a cell surface receptor present on a cell.

Brief Summary Text (159):

Botulinum toxins for use according to the present invention can be stored in lyophilized or vacuum dried form in containers under vacuum pressure. Prior to lyophilization the botulinum toxin can be combined with pharmaceutically acceptable excipients, stabilizers and/or carriers, such as albumin. The lyophilized or vacuum dried material can be reconstituted with saline or water.

Brief Summary Text (160):

The present invention also includes within its scope the use of an implanted controlled release neurotoxin complex so as to provide therapeutic relief from a chronic disorder such as movement disorder. Thus, the neurotoxin can be imbedded within, absorbed, or carried by a suitable polymer matrix which can be implanted or embedded subdermally so as to provide a year or more of delayed and controlled release of the neurotoxin to the desired target tissue. Implantable polymers which permit controlled release of polypeptide drugs are known, and can be used to prepare a botulinum toxin implant suitable for insertion or subdermal attachment. See e.g. Pain 1999;82(1):49-55; Biomaterials 1994;15(5):383-9; Brain Res 1990;515(1-2):309-11 and U.S. Pat. Nos. 6,022,554; 6,011,011; 6,007,843; 5,667,808, and 5,980,945.

Brief Summary Text (161):

Methods for determining the appropriate route of administration and dosage are generally determined on a case by case basis by the attending physician. Such determinations are routine to one of ordinary skill in the art (see for example, Harrison's Principles of Internal Medicine (1998), edited by Anthony Fauci et al., 14.sup.th edition, published by McGraw Hill). Thus, an implant within the scope of the present invention can be surgically inserted by incision at the site of desired effect (i.e. for reduction of a muscle spasm) or the implant can be administered as a suspension, subcutaneously or intramuscularly using a hollow needle implanting gun, for example of the type disclosed in U.S. Pat. No. 4,474,572. The diameter of the needle may be adjusted to correspond to the size of the implant used. Further, an implant within the scope of the present invention can be implanted intracranially so as to provide long term delivery of a therapeutic amount of a neurotoxin to a target brain tissue. Removal of a non-biodegradable implant within the scope of the present invention is not essential once all neurotoxin has been released due to the biocompatible, nonimmunogenic nature of the implant materials used.

Brief Summary Text (162):

It is known that a significant water content of lyophilized tetanus toxoid can cause solid phase aggregation and inactivation of the toxoid once encapsulated within microspheres. Thus, with a 10% (grams of water per 100 grams of protein) tetanus toxoid water content about 25% of the toxin undergoes aggregation, while with a 5% water content only about 5% of the toxoid aggregates. See e.g. Pages 251, Schwendeman S. P. et al., Peptide, Protein, and Vaccine Deliveiy From Implantable Polymeric Systems, chapter 12 (pages 229-267) of Park K., Controlled Drug Delivery Challenges and Strategies, American Chemical Society (1997). Significantly, the manufacturing process for BOTOX.RTM. results in a freeze dried botulinum toxin type A complex which has a moisture content of less than about 3%, at which moisture level nominal solid phase aggregation can be expected.

Brief Summary Text (163):

A general procedure for making a pulsatile, biodegradable botulinum toxin implant is as follows. The implant can comprise from about 25% to about 100% of a polylactide which is a polymer of lactic acid alone. Increasing the amount of lactide in the implant can increases the period of time before which the implant begins to biodegrade, and hence increase the time to pulsatile release of the botulinum toxin from the implant. The implant can also be a copolymer of lactic acid and glycolic acid. The lactic acid can be either in racemic or in optically active form, and can be either soluble in benzene and having an inherent viscosity of from 0.093 (1 g. per 100 ml. in chloroform) to 0.5 (1 g. per 100 ml. in benzene), or insoluble in benzene and having an inherent viscosity of from 0.093 (1 g. per 100 ml in chloroform) to 4 (1 g. per 100 ml in chloroform or dioxin). The implant can also comprise from 0.001% to 50% of a botulinum toxin uniformly dispersed in carrier polymer.

Brief Summary Text (164):

Once implanted the implant begins to absorb water and exhibits two successive and generally distinct phases of neurotoxin release. In the first phase neurotoxin is released through by initial diffusion through aqueous neurotoxin regions which communicate with the exterior surface of the implant. The second phase occurs upon release of neurotoxin consequent to degradation of the biodegradable polymer (i.e. a polylactide). The diffusion phase and the degradation-induced phase are temporally distinct in time. When the implant is placed in an aqueous physiological environment, water diffuses into the polymeric matrix and is partitioned between neurotoxin and polylactide to form aqueous neurotoxin regions. The aqueous neurotoxin regions increase with increasing absorption of water, until the continuity of the aqueous neurotoxin regions reaches a sufficient level to communicate with the exterior surface of the implant. Thus, neurotoxin starts to be released from the implant by diffusion through aqueous polypeptide channels formed from the aqueous neurotoxin regions, while the second phase continues until substantially all of the remaining neurotoxin has been released.

Brief Summary Text (165):

Also within the scope of the present invention is an implant in the form of a suspension for use by injection, prepared by suspending the neurotoxin encapsulated microspheres in a suitabl liquid, such as physiological saline.

Detailed Description Text (4):

Method For Making a Biodegradable Botulinum Toxin Implant

Detailed Description Text (5):

A biodegradable implant comprising botulinum toxin and a suitable carrier polymer can be prepared by dispersing an appropriate amount of a stabilized botulinum toxin preparation (i.e. non-reconstituted BOTOX.RTM.) into a continuous phase consisting of a biodegradable polymer in a volatile organic solvent, such as dichloromethane. Both PLGA and polyanhydrides are insoluble in water and require use of organic solvents in the microencapsulation process.

Detailed Description Text (6):

The polymer is dissolved in an organic solvent such as methylene chloride or ethyl acetate to facilitate microsphere fabrication. The botulinum toxin is then mixed by homogenization or sonication to form a fine dispersion of toxin in polymer/organic solvent, as an emulsion when an aqueous protein solution is used or as a suspension when a solid protein formulation is mixed with the polymer-organic solvent solution. The conventional processes for microsphere formation are solvent evaporation and solvent (coacervation) methods. Microspheres can be formed by mixing the preformed suspension of protein drug with polymer-organic solvent, with water containing an emulsifier (i.e. polyvinyl alcohol). Additional water is then added to facilitate removal of the organic solvent from the microspheres allowing them to harden. The final microspheres are dried to produce a free flowing powder.

Detailed Description Text (7):

The polymer used can be PLA, PGA or a co-polymer thereof. Alternately, a botulinum toxin incorporating polymer can be prepared by emulsifying an aqueous solution of the neurotoxin (i.e. reconstituted BOTOX.RTM.) into the polymer-organic phase (obtaining thereby a W/O emulsion). With either process a high speed stirrer or ultrasound is used to ensure uniform toxin mixing with the polymer. Microparticles 1-50 .mu.m in diameter can be formed by atomizing the emulsion into a stream of hot air, inducing the particle formation through evaporation of the solvent (spray-drying technique). Alternately, particle formation can be achieved by coacervation of the polymer through non-solvent addition, e.g. silicon oil (phase separation technique) or by preparing a W/O/W emulsion (double emulsion technique).

Detailed Description Text (8):

The pH of the casting or other solution in which the botulinum toxin is to be mixed is maintained at pH 4.2-6.8, because at pH above about pH 7 the stabilizing nontoxin proteins can dissociate from the botulinum toxin resulting in gradual loss of toxicity. Preferably, the pH is between about 5-6. Furthermore the temperature of the mixture/solution should not exceed about 35 degrees Celsius, because the toxin can be readily detoxified when in a solution/mixture heated above about 40 degrees Celsius.

Detailed Description Text (10):

A wide range of sizes of botulinum toxin implant microparticles can be made by varying the droplet size, for example, by changing the ultrasonic nozzle diameter. If very large microparticles are desired, the microparticles can be extruded through a syringe directly into the cold liquid. Increasing the viscosity of the polymer solution can also increase microparticle size. The size of the microparticles can be produced by this process, for example microparticles ranging from greater than about 1000 to about 1 micrometers in diameter.

Detailed Description Text (12):

Method For Making a Polyanhydride Botulinum Toxin Implant

Detailed Description Text (13):

A biodegradable polyanhydride polymer can be made as a copolymer of poly-carboxyphenoxypropane and sebacic acid in a ratio of 20:80. Polymer and a botulinum toxin (such as non-reconstituted BOTOX.RTM.) can be co-dissolved in methylene chloride at room temperature and spray-dried into microspheres, using the technique of Example 1. Any remaining methylene chloride can be evaporated in a vacuum desiccator.

Detailed Description Text (14):

Depending upon the implant size desired and hence the amount of botulinum toxin, a suitable amount of the microspheres can be compressed at about 8000 p.s.i. for 5 seconds or at 3000 p.s.i. for 17 seconds in a mold to form implant discs encapsulating the neurotoxin. Thus, the microspheres can be compression molded

pressed into discs 1.4 cm in diameter and 1.0 mm thick, packaged in aluminum foil pouches under nitrogen atmosphere and sterilized by 2.2.times.10.sup.4 Gy gamma irradiation. The polymer permits release of the botulinum toxin over a prolonged period, and it can take more than a year for the polymer to be largely degraded.

Detailed Description Text (16):

Water In Oil Method For Making a Biodegradable Botulinum Toxin Implant

Detailed Description Text (17):

A pulsatile release botulinum toxin implant can be made by dissolving a 80:20 copolymers of polyglycolic acid and the polylactic acid can in 10% w/v of dichloromethane at room temperature with gentle agitation. A water-in-oil type emulsion can then be made by adding 88 parts of the polymer solution to 1 part of a 1:5 mixture of Tween 80 (polyoxyethylene 20 sorbitan monooleate, available from Acros Organics N.V., Fairlawn, N.J.) and Span 85 (sorbitan trioleate) and 11 parts of an aqueous mixture of 75 units of BOTOX.RTM. (botulinum toxin type A complex) and Quil A (adjuvant). The mixture is agitated using a high-speed blender and then immediately spray-dried using a Drytec Compact Laboratory Spray Dryer equipped with a 60/100/120 nozzle at an atomizing pressure of 15 psi and an inlet temperature of 65 degrees C. The resultant microspheres have a diameter of about 20 .mu.m diameter and are collected as a free-flowing powder. Traces of remaining organic solvent are removed by vacuum evaporation.

Detailed Description Text (19):

Reduced Temperature Method For a Biodegradable Pulsatile Botulinum Toxin Implant

Detailed Description Text (20):

A pulsatile release botulinum toxin delivery system can be made at a low temperature temperature so as to inhibit toxin denaturation as follows. 0.3 g of PLGA/ml of methylene chloride or ethyl acetate is mixed with 0.1 ml of neurotoxin solution/ml of the polymer-organic solution at a reduced temperature (2-8 degrees C.). A first set of botulinum toxin incorporating microspheres made, as set forth in Example 1 (the polymer solution is formed by dissolving the polymer in methylene chloride), from a 75:25 lactide:glycolide polymer with an inherent viscosity (dL/g) of about 0.62 (available form MTI) can degrade in vivo, and hence exhibit a pulsed release of the botulinum toxin, at about ninety days post implantation and extending over 2-2-4 weeks. A second set of, botulinum toxin incorporating microspheres made, as previously set forth (the polymer solution is formed by dissolving the polymer in ethyl acetate), from a 100:0 lactide:glycolide polymer with an inherent viscosity of about 0.22 (available form MTI) can degrade in vivo, and hence exhibit a burst release of the botulinum toxin, at about one hundred and eighty days post implantation. A third set of, botulinum toxin incorporating microspheres made, as previously set forth (the polymer solution is formed by dissolving the polymer in methylene chloride, from a 95:5 poly(DL-lactide):glycolide polymer, can degrade in vivo, and hence exhibit a burst release of the botulinum toxin, at about two hundred and seventy days post implantation. A fourth set of botulinum toxin incorporating microspheres made, as previously set forth (the polymer solution is formed by dissolving the polymer in methylene chloride), from a 100:0 poly(DL-lactide):glycolide polymer can degrade in vivo, and hence exhibit a burst release of the botulinum toxin, at about twelve months post implantation. Polymers can be obtained from Medisorb Technologies International (MTI).

Detailed Description Text (21):

A suspension or compression molded pellet which combines the four specified sets of botulinum toxin encapsulated microspheres can exhibit pulsatile release the neurotoxin. Local administration of botulinum toxin at the time of implantation (i.e. day zero) is provided by the initial burst release from the implanted microspheres.

Detailed Description Text (22):

Compositions and methods according to the invention disclosed herein has many advantages, including the following: 1. a single implant can be used to provide therapeutically effective continuous or pulsatile administration of a neurotoxin over a period of one year or longer. 2. the neurotoxin is delivered to a localized tissue area without a significant amount of neurotoxin appearing systemically. 3. reduced need for patient follow up care. 4. reduced need for periodic injections of neurotoxin to treat a condition, such as a neuromuscular disorder. 5. increased patient comfort due to the reduced number of injections required. 6. improved patient compliance.

Detailed Description Text (23):

An advantage of the present controlled release formulations for neurotoxins include long term, consistent therapeutic levels of neurotoxin at the target tissue. The advantages also include increased patient compliance and acceptance by reducing the required number of injections.

Detailed Description Text (25):

Although the present invention has been described in detail with regard to certain preferred methods, other embodiments, versions, and modifications within the scope of the present invention are possible. For example, a wide variety of neurotoxins can be effectively used in the methods of the present invention. Additionally, the present invention includes local (i.e. intramuscular, intraglandular, subcutaneous, and intracranial) administration methods wherein two or more neurotoxins, such as two or more botulinum toxins, are administered concurrently or consecutively via implant. For example, botulinum toxin type A can be administered via implant until a loss of clinical response or neutralizing antibodies develop, followed by administration via implant of a botulinum toxin type B or E. Alternately, a combination of any two or more of the botulinum serotypes A-G can be locally administered to control the onset and duration of the desired therapeutic result. Furthermore, non-neurotoxin compounds can be administered prior to, concurrently with or subsequent to administration of the neurotoxin via implant so as to provide an adjunct effect such as enhanced or a more rapid onset of denervation before the neurotoxin, such as a botulinum toxin, begins to exert its therapeutic effect.

Detailed Description Text (26):

The present invention also includes within its scope the use of a neurotoxin, such as a botulinum toxin, in the preparation of a medicament, such as a controlled release implant, for the treatment of a movement disorder, and/or a disorder influenced by cholinergic innervation, by local administration via the implant of the neurotoxin.

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CLAIMS:

1. A pulsatile release botulinum toxin delivery system, comprising: (a) a carrier; (b) a botulinum toxin associated with the carrier, thereby forming a pulsatile release botulinum toxin delivery system, wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a plurality of pulses in vivo upon subdermal implantation of the delivery system in a human patient without a significant immune system response and wherein the carrier is comprised of a biodegradable material selected from the group consisting of polymers of poly(lactides), poly(glycolides), collagens, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(lactic acid-co-glycolic acid)s, polycaprolactone, polycarbonates, polyesteramides, polyanhydrides, poly(amino acids), polyorthoesters, polycyanoacrylates, poly(p-dioxanone), poly(alkylene oxalates), biodegradable polyurethanes, blends and copolymers thereof.
3. The delivery system of claim 1, wherein substantial amounts of the botulinum toxin has not be transformed into a botulinum toxoid prior to association of the botulinum toxin with the carrier.
4. The delivery system of claim 1, wherein significant amounts of the botulinum toxin associated with the carrier have a toxicity which is substantially unchanged relative to the toxicity of the botulinum toxin prior to association of the botulinum toxin with the carrier.
6. The delivery system of claim 1, wherein the botulinum toxin can be released from the carrier over of a period of time of from about 10 days to about 6 years.
7. The delivery system of claim 1, wherein the botulinum toxin is selected from the group consisting of botulinum toxin types A, B, C.sub.1, D, E, F and G.
8. The delivery system of claim 1, wherein the botulinum toxin is a botulinum toxin type A.
9. The delivery system of claim 1, wherein the quantity of the botulinum toxin associated with the carrier is between about 1 unit and about 50,000 units of the

botulinum toxin.

10. The delivery system of claim 1, wherein the quantity of the botulinum toxin is between about 10 units and about 2,000 units of a botulinum toxin type A.

11. The delivery system of claim 1, wherein the quantity of the botulinum toxin is between about 100 units and about 30,000 units of a botulinum toxin type B.

12. A controlled release system, comprising: (a) a biodegradable polymer; (b) between about 10 units and about 100,000 units of a botulinum toxin encapsulated by the polymer carrier, thereby forming a controlled release system, wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a pulsatile manner in vivo upon subdermal implantation of the controlled release system in a human patient over a prolonged period of time extending from about 2 months to about 5 years without a significant immune system response and wherein the carrier is comprised of a biodegradable material selected from the group consisting of polymers of poly(lactides), poly(glycolides), collagens, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(lactic acid-co-glycolic acid)s, polycaprolactone, polycarbonates, polyesteramides, polyanhydrides, poly(amino acids), polyorthoesters, polycyanoacrylates, poly(p-dioxanone), poly(alkylene oxalates), biodegradable polyurethanes, blends and copolymers thereof.

13. A method for making a controlled release system, the method comprising the steps of: (a) dissolving a polymer in a solvent to form a polymer solution; (b) mixing or dispersing a botulinum toxin in the polymer solution to form a polymer-botulinum toxin mixture, and; (c) allowing the polymer-botulinum toxin mixture to set or cure, thereby making a controlled release system for pulsatile release of the botulinum toxin without a significant immune system response, wherein the polymer is comprised of a biodegradable material selected from the group consisting of polymers of poly(lactides), poly(glycolides), collagens, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(lactic acid-co-glycolic acid)s, polycaprolactone, polycarbonates, polyesteramides, polyanhydrides, poly(amino acids), polyorthoesters, polycyanoacrylates, poly(p-dioxanone), poly(alkylene oxalates), biodegradable polyurethanes, blends and copolymers thereof.

15. A pulsatile release botulinum toxin delivery system, comprising: (a) a carrier comprised of a biodegradable material selected from the group consisting of polymers of poly(lactides), poly(glycolides), collagens, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(lactic acid-co-glycolic acid)s, polycaprolactone, polycarbonates, polyesteramides, polyanhydrides, poly(amino acids), polyorthoesters, polycyanoacrylates, poly(p-dioxanone), poly(alkylene oxalates), biodegradable polyurethanes, blends and copolymers thereof; (b) a stabilized botulinum toxin associated with the carrier, thereby forming a pulsatile release botulinum toxin delivery system, wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a plurality of pulses in vivo upon subdermal implantation of the delivery system in a human patient without a significant immune system response.

16. The delivery system of claim 15, wherein the carrier comprises a plurality of discrete sets of polymeric, botulinum toxin incorporating microspheres, wherein each set of polymers has a different polymeric composition.

17. The delivery system of claim 15, wherein the botulinum toxin comprises: (a) a first element comprising a binding element able to specifically bind to a neuronal cell surface receptor under physiological conditions, (b) a second element comprising a translocation element able to facilitate the transfer of a polypeptide across a neuronal cell membrane, and (c) a third element comprising a therapeutic element able, when present in the cytoplasm of a neuron, to inhibit exocytosis of acetylcholine from the neuron.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Abstract	Claims	KWIC	Draw. De
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L5: Entry 6 of 19

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TITLE: Recombinant toxin fragments

Abstract Text (1):

A polypeptide has first and second domains which enable the polypeptide to be translocated into a target cell or which increase the solubility of the polypeptide, or both, and further enable the polypeptide to cleave one or more vesicle or plasma-membrane associated proteins essential to exocytosis. The polypeptide thus combines useful properties of a clostridial toxin, such as a botulinum or tetanus toxin, without the toxicity associated with the natural molecule. The polypeptide can also contain a third domain that targets it to a specific cell, rendering the polypeptide useful in inhibition of exocytosis in target cells. Fusion proteins comprising the polypeptide, nucleic acids encoding the polypeptide and methods of making the polypeptide are also provided. Controlled activation of the polypeptide is possible and the polypeptide can be incorporated into vaccines and toxin assays.

Brief Summary Text (4):

The clostridial neurotoxins are potent inhibitors of calcium-dependent neurotransmitter secretion in neuronal cells. They are currently considered to mediate this activity through a specific endoproteolytic cleavage of at least one of three vesicle or pre-synaptic membrane associated proteins VAMP, syntaxin or SNAP-25 which are central to the vesicle docking and membrane fusion events of neurotransmitter secretion. The neuronal cell targeting of tetanus and botulinum neurotoxins is considered to be a receptor mediated event following which the toxins become internalised and subsequently traffic to the appropriate intracellular compartment where they effect their endopeptidase activity.

Brief Summary Text (5):

The clostridial neurotoxins share a common architecture of a catalytic L-chain (LC, ca 50 kDa) disulphide linked to a receptor binding and translocating H-chain (HC, ca 100 kDa). The HC polypeptide is considered to comprise all or part of two distinct functional domains. The carboxy-terminal half of the HC (ca 50 kDa), termed the H.sub.C domain, is involved in the high affinity, neurospecific binding of the neurotoxin to cell surface receptors on the target neuron, whilst the amino-terminal half, termed the H.sub.N domain (ca 50 kDa), is considered to mediate the translocation of at least some portion of the neurotoxin across cellular membranes such that the functional activity of the LC is expressed within the target cell. The H.sub.N domain also has the property, under conditions of low pH, of forming ion-permeable channels in lipid membranes, this may in some manner relate to its translocation function.

Brief Summary Text (6):

For botulinum neurotoxin type A (BoNT/A) these domains are considered to reside within amino acid residues 872-1296 for the H.sub.C, amino acid residues 449-871

for the H.sub.N and residues 1-448 for the LC. Digestion with trypsin effectively degrades the H.sub.C domain of the BoNT/A to generate a non-toxic fragment designated LH.sub.N, which is no longer able to bind to and enter neurons (FIG. 1). The LH.sub.N fragment so produced also has the property of enhanced solubility compared to both the parent holotoxin and the isolated LC.

Brief Summary Text (7):

It is therefore possible to provide functional definitions of the domains within the neurotoxin molecule, as follows:

Brief Summary Text (8):

(A) Clostridial Neurotoxin Light Chain: a metalloprotease exhibiting high substrate specificity for vesicle and/or plasma-membrane associated proteins involved in the exocytotic process. In particular, it cleaves one or more of SNAP-25, VAMP (synaptobrevin/cellubrevin) and syntaxin.

Brief Summary Text (9):

(B) Clostridial Neurotoxin Heavy Chain H.sub.N Domain: a portion of the heavy chain which enables translocation of that portion of the neurotoxin molecule such that a functional expression of light chain activity occurs within a target cell. the domain responsible for translocation of the endopeptidase activity, following binding of neurotoxin to its specific cell surface receptor via the binding domain, into the target cell. the domain responsible for formation of ion-permeable pores in lipid membranes under conditions of low pH. the domain responsible for increasing the solubility of the entire polypeptide compared to the solubility of light chain alone.

Brief Summary Text (10):

(C) Clostridial Neurotoxin Heavy Chain H.sub.C Domain. a portion of the heavy chain which is responsible for binding of the native holotoxin to cell surface receptor (s) involved in the intoxicating action of clostridial toxin prior to internalisation of the toxin into the cell.

Brief Summary Text (11):

The identity of the cellular recognition markers for these toxins is currently not understood and no specific receptor species have yet been identified although Kozaki et al. have reported that synaptotagmin may be the receptor for botulinum neurotoxin type B. It is probable that each of the neurotoxins has a different receptor.

Brief Summary Text (12):

It is desirable to have positive controls for toxin assays, to develop clostridial toxin vaccines and to develop therapeutic agents incorporating desirable properties of clostridial toxin.

Brief Summary Text (14):

The present invention seeks to overcome or at least ameliorate problems associated with production and handling of clostridial toxin.

Brief Summary Text (16):

Accordingly, the invention provides a polypeptide comprising first and second domains, wherein said first domain is adapted to cleave one or more vesicle or plasma-membrane associated proteins essential to neuronal exocytosis and wherein said second domain is adapted (i) to translocate the polypeptide into the cell or (ii) to increase the solubility of the polypeptide compared to the solubility of the first domain on its own or (iii) both to translocate the polypeptide into the cell and to increase the solubility of the polypeptide compared to the solubility of the first domain on its own, said polypeptide being free of clostridial neurotoxin and free of any clostridial neurotoxin precursor that can be converted into toxin by proteolytic action. Accordingly, the invention may thus provide a

single polypeptide chain containing a domain equivalent to a clostridial toxin light chain and a domain providing the functional aspects of the H.sub.N of a clostridial toxin heavy chain, whilst lacking the functional aspects of a clostridial toxin H.sub.C domain.

Brief Summary Text (18):

For the purposes of the invention, the functional property or properties of the H.sub.N of a clostridial toxin heavy chain that are required to be exhibited by the second domain of the polypeptide of the invention are either (i) translocation of the polypeptide into a cell, or (ii) increasing solubility of the polypeptide compared to solubility of the first domain on its own or (iii) both (i) and (ii). References hereafter to a H.sub.N domain or to the functions of a H.sub.N domain are references to this property or properties. The second domain is not required to exhibit other properties of the H.sub.N domain of a clostridial toxin heavy chain.

Brief Summary Text (19):

A polypeptide of the invention can thus be soluble but lack the translocation function of a native toxin--this is of use in providing an immunogen for vaccinating or assisting to vaccinate an individual against challenge by toxin. In a specific embodiment of the invention described in an example below a polypeptide designated LH.sub.423 /A elicited neutralising antibodies against type A neurotoxin. A polypeptide of the invention can likewise thus be relatively insoluble but retain the translocation function of a native toxin--this is of use if solubility is imparted to a composition made up of that polypeptide and one or more other components by one or more of said other components.

Brief Summary Text (20):

The first domain of the polypeptide of the invention cleaves one or more vesicle or plasma-membrane associated proteins essential to the specific cellular process of exocytosis, and cleavage of these proteins results in inhibition of exocytosis, typically in a non-cytotoxic manner. The cell or cells affected are not restricted to a particular type or subgroup but can include both neuronal and non-neuronal cells. The activity of clostridial neurotoxins in inhibiting exocytosis has, indeed, indeed, been observed almost universally in eukaryotic cells expressing a relevant cell surface receptor, including such diverse cells as from *Aplysia* (sea slug), *Drosophila* (fruit fly) and mammalian nerve cells, and the activity of the first domain is to be understood as including a corresponding range of cells.

Brief Summary Text (22):

In a polypeptide according to the invention, said first domain preferably comprises a clostridial toxin light chain or a fragment or variant of a clostridial toxin light chain. The fragment is optionally an N-terminal, or C-terminal fragment of the light chain, or is an internal fragment, so long as it substantially retains the ability to cleave the vesicle or plasma-membrane associated protein essential to exocytosis. The minimal domains necessary for the activity of the light chain of clostridial toxins are described in J. Biol. Chem., Vol.267, No. 21, July 1992, pages 14721-14729. The variant has a different peptide sequence from the light chain or from the fragment, though it too is capable of cleaving the vesicle or plasma-membrane associated protein. It is conveniently obtained by insertion, deletion and/or substitution of a light chain or fragment thereof. In embodiments of the invention described below a variant sequence comprises (i) an N-terminal extension to a clostridial toxin light chain or fragment (ii) a clostridial toxin light chain or fragment modified by alteration of at least one amino acid (iii) a C-terminal extension to a clostridial toxin light chain or fragment, or (iv) combinations of 2 or more of (i)-(iii).

Brief Summary Text (24):

The first domain preferably exhibits endopeptidase activity specific for a substrate selected from one or more of SNAP-25, synaptobrevin/VAMP and syntaxin. The clostridial toxin is preferably botulinum toxin or tetanus toxin.

Brief Summary Text (25):

In an embodiment of the invention described in an example below, the toxin light chain and the portion of the toxin heavy chain are of botulinum toxin type A. In a further embodiment of the invention described in an example below, the toxin light chain and the portion of the toxin heavy chain are of botulinum toxin type B. The polypeptide optionally comprises a light chain or fragment or variant of one toxin type and a heavy chain or fragment or variant of another toxin type.

Brief Summary Text (26):

In a polypeptide according to the invention said second domain preferably comprises a clostridial toxin heavy chain H.sub.N portion or a fragment or variant of a clostridial toxin heavy chain H.sub.N portion. The fragment is optionally an N-terminal or C-terminal or internal fragment, so long as it retains the function of the H.sub.N domain. Teachings of regions within the H.sub.N responsible for its function are provided for example in Biochemistry 1995, 34, pages 15175-15181 and Eur. J. Biochem, 1989, 185, pages 197-203. The variant has a different sequence from the H.sub.N domain or fragment, though it too retains the function of the H.sub.N domain. It is conveniently obtained by insertion, deletion and/or substitution of a H.sub.N domain or fragment thereof. In embodiments of the invention, described below, it comprises (i) an N-terminal extension to a H.sub.N domain or fragment, (ii) a C-terminal extension to a H.sub.N domain or fragment, (iii) a modification to a H.sub.N domain or fragment by alteration of at least one amino acid, or (iv) combinations of 2 or more of (i)-(iii). The clostridial toxin is preferably botulinum toxin or tetanus toxin.

Brief Summary Text (27):

The invention also provides a polypeptide comprising a clostridial neurotoxin light chain and a N-terminal fragment of a clostridial neurotoxin heavy chain, the fragment preferably comprising at least 423 of the N-terminal amino acids of the heavy chain of botulinum toxin type A, 417 of the N-terminal amino acids of the heavy chain of botulinum toxin type B or the equivalent number of N-terminal amino acids of the heavy chain of other types of clostridial toxin such that the fragment possesses an equivalent alignment of homologous amino acid residues.

Brief Summary Text (28):

These polypeptides of the invention are thus not composed of two or more polypeptides, linked for example by di-sulphide bridges into composite molecules. Instead, these polypeptides are single chains and are not active or their activity is significantly reduced in an in vitro assay of neurotoxin endopeptidase activity.

Brief Summary Text (30):

In a specific embodiment of the invention described in an example below, there is provided a polypeptide lacking a portion designated H.sub.C of a clostridial toxin heavy chain. This portion, seen in the naturally produced toxin, is responsible for binding of toxin to cell surface receptors prior to internalisation of the toxin. This specific embodiment is therefore adapted so that it can not be converted into active toxin, for example by the action of a proteolytic enzyme. The invention thus also provides a polypeptide comprising a clostridial toxin light chain and a fragment of a clostridial toxin heavy chain, said fragment being not capable of binding to those cell surface receptors involved in the intoxicating action of clostridial toxin, and it is preferred that such a polypeptide lacks an intact portion designated H.sub.C of a clostridial toxin heavy chain.

Brief Summary Text (31):

In further embodiments of the invention there are provided compositions containing a polypeptide comprising a clostridial toxin light chain and a portion designated H.sub.N of a clostridial toxin heavy chain, and wherein the composition is free of clostridial toxin and free of any clostridial toxin precursor that may be converted

into clostridial toxin by the action of a proteolytic enzyme. Examples of these compositions include those containing toxin light chain and H.sub.N sequences of botulinum toxin types A, B, C.sub.1, D, E, F and G.

Brief Summary Text (32):

The polypeptides of the invention are conveniently adapted to bind to, or include, a ligand for targeting to desired cells. The polypeptide optionally comprises a sequence that binds to, for example, an immunoglobulin. A suitable sequence is a tandem repeat synthetic IgG binding domain derived from domain B of Staphylococcal protein A. Choice of immunoglobulin specificity then determines the target for a polypeptide-immunoglobulin complex. Alternatively, the polypeptide comprises a non-clostridial sequence that binds to a cell surface receptor, suitable sequences including insulin-like growth factor-1 (IGF-1) which binds to its specific receptor on particular cell types and the 14 amino acid residue sequence from the carboxy-terminus of cholera toxin A subunit which is able to bind the cholera toxin B subunit and thence to GM1 gangliosides. A polypeptide according to the invention thus, optionally, further comprises a third domain adapted for binding of the polypeptide to a cell.

Brief Summary Text (35):

As noted above, by proteolytic treatment, for example using trypsin, of a polypeptide of the invention it is possible to induce endopeptidase activity in the treated polypeptide. A third aspect of the invention provides a composition comprising a derivative of a clostridial toxin, said derivative retaining at least 10% of the endopeptidase activity of the clostridial toxin, said derivative further being non-toxic in vivo due to its inability to bind to cell surface receptors, and wherein the composition is free of any component, such as toxin or a further toxin derivative, that is toxic in vivo. The activity of the derivative preferably approaches that of natural toxin, -and is thus preferably at least 30% and most preferably at least 60% of natural toxin. The overall endopeptidase activity of the composition will, of course, also be determined by the amount of the derivative that is present.

Brief Summary Text (36):

While it is known to treat naturally produced clostridial toxin to remove the Hc domain, this treatment does not totally remove toxicity of the preparation, instead some residual toxin activity remains. Natural toxin treated in this way is therefore still not entirely safe. The composition of the invention, derived by treatment of a pure source of polypeptide advantageously is free of toxicity, and can conveniently be used as a positive control in a toxin assay, as a vaccine against clostridial toxin or for other purposes where it is essential that there is no residual toxicity in the composition.

Brief Summary Text (39):

In one embodiment of this aspect of the invention, a DNA sequence provided to code for the polypeptide or fusion protein is not derived from native clostridial sequences, but is an artificially derived sequence not preexisting in nature.

Brief Summary Text (40):

A specific DNA (SEQ ID NO: 1) described in more detail below encodes a polypeptide or a fusion protein comprising nucleotides encoding residues 1-871 of a botulinum toxin type A. Said polypeptide comprises the light chain domain and the first 423 amino acid residues of the amino terminal portion of a botulinum toxin type A heavy chain. This recombinant product is designated LH.sub.423 /A (SEQ ID NO: 2).

Brief Summary Text (41):

In a second embodiment of this aspect of the invention a DNA sequence which codes for the polypeptide or fusion protein is derived from native clostridial sequences but codes for a polypeptide or fusion protein not found in nature.

Brief Summary Text (42):

A specific DNA (SEQ ID NO: 19) described in more detail below encodes a polypeptide or a fusion protein and comprises nucleotides encoding residues 1-1171 of a botulinum toxin type B. Said polypeptide comprises the light chain domain and the first 728 amino acid residues of the amino terminal protein of a botulinum type B heavy chain. This recombinant product is designated LH728/B (SEQ ID NO: 20).

Brief Summary Text (43):

The invention thus also provides a method of manufacture of a polypeptide comprising expressing in a host cell a DNA according to the third aspect of the invention. The host cell is suitably not able to cleave a polypeptide or fusion protein of the invention so as to separate light and heavy toxin chains; for example, a non-clostridial host.

Brief Summary Text (45):

The LH.sub.N /A derived from dichain native toxin requires extended digestion with trypsin to remove the C-terminal 1/2 of the heavy chain, the H.sub.C domain. The loss of this domain effectively renders the toxin inactive in vivo by preventing its interaction with host target cells. There is, however, a residual toxic activity which may indicate a contaminating, trypsin insensitive, form of the whole type A neurotoxin.

Brief Summary Text (48):

One application of the recombinant polypeptides of the invention will be as a reagent component for synthesis of therapeutic molecules, such as disclosed in WO-A-94/21300. The recombinant product will also find application as a non-toxic standard for the assessment and development of in vitro assays for detection of functional botulinum or tetanus neurotoxins either in foodstuffs or in environmental environmental samples, for example as disclosed in EP-A-0763131.

Brief Summary Text (51):

The LH.sub.N enzymatically produced from native BoNT/A is an efficient immunogen and thus the recombinant form with its total divorce from any full length neurotoxin represents a vaccine component. The recombinant product may serve as a basal reagent for creating defined protein modifications in support of any of the above areas.

Brief Summary Text (52):

Recombinant constructs are assigned distinguishing names on the basis of their amino acid sequence length and their Light Chain (L-chain, L) and Heavy Chain (H-chain, H) content as these relate to translated DNA sequences in the public domain or specifically to SEQ ID NO: 2 and SEQ ID NO: 20. The `LH` designation is followed by `/X` where `X` denotes the corresponding clostridial toxin serotype or class, e.g. `A` for botulinum neurotoxin type A or `TeTx` for tetanus toxin. Sequence variants from that of the native toxin polypeptide are given in parenthesis in standard format, namely the residue position number prefixed by the residue of the native sequence and suffixed by the residue of the variant.

Brief Summary Text (53):

Subscript number prefixes indicate an amino-terminal (N-terminal) extension, or where negative a deletion, to the translated sequence. Similarly, subscript number suffixes indicate a carboxy terminal (C-terminal) extension or where negative numbers are used, a deletion. Specific sequence inserts such as protease cleavage sites are indicated using abbreviations, e.g. Factor Xa is abbreviated to FXa. L-chain C-terminal suffixes and H-chain N-terminal prefixes are separated by a `/` to indicate the predicted junction between the L and H-chains. Abbreviations for engineered ligand sequences are prefixed or suffixed to the clostridial L-chain or H-chain corresponding to their position in the translation product.

Brief Summary Text (54):

Following this nomenclature, LH.sub.423 /A=SEQ ID NO: 2, containing the entire L-chain and 423 amino acids of the H-chain of botulinum neurotoxin type A; .sub.2 LH.sub.423 /A=a variant of this molecule, containing a two amino acid extension to the N-terminus of the L-chain; .sub.2 L.sub./2 H.sub.423 /A=a further variant in which the molecule contains a two amino acid extension on the N-terminus of both the L-chain and the H-chain; .sub.2 L.sub.FXa/2 H.sub.423 /A=a further variant containing a two amino acid extension to the N-terminus of the L-chain, and a Factor Xa cleavage sequence at the C-terminus of the L-chain which, after cleavage of the molecule with Factor Xa leaves a two amino acid N-terminal extension to the H-chain component; and .sub.2 L.sub.FXa/2 H.sub.423 /A-IGF-1=a variant of this molecule which has a further C-terminal extension to the H-chain, in this example the insulin-like growth factor 1 (IGF-1) sequence.

Drawing Description Text (2):

FIG. 1 shows a schematic representation of the domain structure of botulinum neurotoxin type A (BoNT/A);

Detailed Description Text (4):

A 2616 base pair, double stranded gene sequence (SEQ ID NO: 1) has been assembled from a combination of synthetic, chromosomal and polymerase-chain-reaction generated DNA (FIG. 2). The gene codes for a polypeptide of 871 amino acid residues corresponding to the entire light-chain (LC, 448 amino acids) and 423 residues of the amino terminus of the heavy-chain (Hc) of botulinum neurotoxin type A. This recombinant product is designated the LH.sub.423 /A fragment (SEQ ID NO: 2).

Detailed Description Text (6):

The first 918 base pairs of the recombinant gene were synthesised by concatenation of short oligonucleotides to generate a coding sequence with an E. coli codon bias. Both DNA strands in this region were completely synthesised as short overlapping oligonucleotides which were phosphorylated, annealed and ligated to generate the full synthetic region ending with a unique KpnI restriction site. The remainder of the LH.sub.423 /A coding sequence was PCR amplified from total chromosomal DNA from Clostridium botulinum and annealed to the synthetic portion of the gene.

Detailed Description Text (7):

The internal PCR amplified product sequences were then deleted and replaced with the native, fully sequenced, regions from clones of C. botulinum chromosomal origin to generate the final gene construct. The final composition is synthetic DNA (bases 1-913), polymerase amplified DNA (bases 914-1138 and 1976-2616) and the remainder is of C. botulinum chromosomal origin (bases 1139-1975). The assembled gene was then then fully sequenced and cloned into a variety of E.coli plasmid vectors for expression analysis.

Detailed Description Text (15):

In yet another variant a gene has been produced which contains a Eco 47 III restriction site between nucleotides 1344 and 1345 of the gene sequence given in (SEQ ID NO: 1). This modification provides a restriction site at the position in the gene representing the interface of the heavy and light chains in native neurotoxin, and provides the capability to make insertions at this point using standard restriction enzyme methodologies known to those skilled in the art. It will also be obvious to those skilled in the art that any one of a number of restriction sites could be so employed, and that the Eco 47 III insertion simply exemplifies this approach. Similarly, it would be obvious for one skilled in the art that insertion of a restriction site in the manner described could be performed on any gene of the invention. The gene described, when expressed, codes for a polypeptide, L.sub./4 H.sub.423 1A (SEQ ID NO: 10), which contains an additional four amino acids between amino acids 448 and 449 of LH.sub.423 /A at a position equivalent to the amino terminus of the heavy chain of native BoNT/A.

Detailed Description Text (18):

Specific examples described are: (1) L.sub.FXa/3 H.sub.423 /A-IGF-1 (SEQ ID NO: 14), in which the carboxy-terminal domain has a sequence equivalent to that of insulin-like growth factor-1 (IGF-1) and is able to bind to the insulin-like growth factor receptor with high affinity; (2) L.sub.FXa/3 H.sub.423 /A-CtxA14 (SEQ ID NO: 16), in which the carboxy-terminal domain has a sequence equivalent to that of the 14 amino acids from the carboxy-terminus of the A-subunit of cholera toxin (CtxA) and is thereby able to interact with the cholera toxin B-subunit pentamer; and (3) L.sub.FXa/3 H.sub.423 /A-ZZ (SEQ ID NO: 18), in which the carboxy-terminal domain is a tandem repeating synthetic IgG binding domain. This variant also exemplifies another modification applicable to the current invention, namely the inclusion in the gene of a sequence coding for a protease cleavage site located between the end of the clostridial heavy chain sequence and the sequence coding for the binding ligand. Specifically in this example a sequence is inserted at nucleotides 2650 to 2666 coding for a genenase cleavage site. Expression of this gene produces a polypeptide which has the desired protease sensitivity at the interface between the domain providing H.sub.N function and the binding domain. Such a modification enables selective removal of the C-terminal binding domain by treatment of the polypeptide with the relevant protease.

Detailed Description Text (22):

FIG. 10 is a diagrammatic representation of an expression product exemplifying features described in this example. Specifically, it illustrates a single polypeptide incorporating a domain equivalent to the light chain of botulinum neurotoxin type A and a domain equivalent to the H.sub.N domain of the heavy chain of botulinum neurotoxin type A with a N-terminal extension providing an affinity purification domain, namely GST, and a C-terminal extension providing a ligand binding domain, namely an igG binding domain. The domains of the polypeptide are spatially separated by specific protease cleavage sites enabling selective enzymatic separation of domains as exemplified in the Figure. This concept is more specifically depicted in FIG. 11 where the various protease sensitivities are defined for the purpose of example.

Detailed Description Text (24):

The LC of botulinum neurotoxin type A exerts a zinc-dependent endopeptidase activity on the synaptic vesicle associated protein SNAP-25 which it cleaves in a specific manner at a single peptide bond. The .sub.2 LH.sub.423 /A (Q.sub.2 E,N.sub.26 K,A.sub.27 Y) (SEQ ID NO: 6) cleaves a synthetic SNAP-25 substrate in vitro under the same conditions as the native toxin (FIG. 3). Thus, the modification of the polypeptide sequence of .sub.2 LH.sub.423 /A (Q.sub.2 E,N.sub.26 K,A.sub.27 Y) relative to the native sequence and within the minimal functional LC domains does not prevent the functional activity of the LC domains.

Detailed Description Text (25):

This activity is dependent on proteolytic modification of the recombinant GST-.sub.2 LH.sub.423 /A (Q.sub.2 E,N.sub.26 K,A.sub.27 Y) to convert the single chain polypeptide product to a disulphide linked dichain species. This is currently done using the proteolytic enzyme trypsin. The recombinant product (100-600 .mu.g/ml) is incubated at 37.degree. C. for 10-50 minutes with trypsin (10 .mu.g/ml) in a solution containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na.sub.2 HPO.sub.4, 1.8 mM KH.sub.2 PO.sub.4, pH 7.3. The reaction is terminated by addition of a 100-fold molar excess of trypsin inhibitor. The activation by trypsin generates a disulphide linked dichain species as determined by polyacrylamide gel electrophoresis and immunoblotting analysis using polyclonal anti-botulinum neurotoxin type A antiserum.

Detailed Description Text (28):

As a further exemplification of this invention a number of gene sequences have been assembled coding for polypeptides corresponding to the entire light-chain and varying numbers of residues from the amino terminal end of the heavy chain of botulinum neurotoxin type B. In this exemplification of the disclosure the gene

sequences assembled were obtained from a combination of chromosomal and polymerase-chain-reaction generated DNA, and therefore have the nucleotide sequence of the equivalent regions of the natural genes, thus exemplifying the principle that the substance of this disclosure can be based upon natural as well as a synthetic gene sequences.

Detailed Description Text (30):

A gene has been assembled coding for a polypeptide of 1171 amino acids corresponding to the entire light-chain (443 amino acids) and 728 residues from the amino terminus of the heavy chain of neurotoxin type B. Expression of this gene produces a polypeptide, LH.sub.728 /B (SEQ ID NO: 20), which lacks the specific neuronal binding activity of full length BoNT/B.

Detailed Description Text (36):

For the first sequence, six oligonucleotides representing the first (5') 268 nucleotides of the native sequence for botulinum toxin type B were synthesised. For the second sequence 23 oligonucleotides representing internal sequence nucleotides 691-1641 of the native sequence for botulinum toxin type B were synthesised. The oligonucleotides ranged from 57-73 nucleotides in length. Overlapping regions, 17-20 nucleotides, were designed to give melting temperatures in the range 52-56.degree. C. In addition, terminal restriction endonuclease sites of the synthetic products were constructed to facilitate insertion of these products into the exact corresponding region of the native sequence. The 268 bp 5' synthetic sequence has been incorporated into the gene shown in SEQ ID NO: 21 in place of the original first 268 bases (and is shown in SEQ ID NO: 27). Similarly the sequence could be inserted into other genes of the examples.

Detailed Description Text (40):

For immunisation, 20 .mu.g of GST-.sub.2 LH.sub.423 /A in a 1.0 ml volume of water-in-oil emulsion (1:1 vol:vol) using Freund's complete (primary injections only) or Freund's incomplete adjuvant was administered into guinea pigs via two sub-cutaneous dorsal injections. Three injections at 10 day intervals were given (day 1, day 10 and day 20) and antiserum collected on day 30. The antisera were shown by ELISA to be immunoreactive against native botulinum neurotoxin type A and to its derivative LH.sub.N /A. Antisera which were botulinum neurotoxin reactive at a dilution of 1:2000 were used for evaluation of neutralising efficacy in mice. For neutralisation assays 0.1 ml of antiserum was diluted into 2.5 ml of gelatine phosphate buffer (GPB; Na.sub.2 HPO.sub.4 anhydrous 10 g/l, gelatin (Difco) 2 g/l, pH 6.5-6.6) containing a dilution range from 0.5 .mu.g (5.times.10.sup.-6 g) to 5 picograms (5.times.10.sup.-12 g). Aliquots of 0.5 ml were injected into mice intraperitoneally and deaths recorded over a 4 day period. The results are shown in Table 1 and Table 2. It can clearly be seen that 0.5 ml of 1:40 diluted anti-GST-.sub.2 LH.sub.423 /A antiserum can protect mice against intraperitoneal challenge with botulinum neurotoxin in the range 5 pg-50 ng (1-10,000 mouse LD50; 1 mouse LD50=5 pg).

Detailed Description Text (43):

As an exemplification of the expression of a nucleic acid coding for a LH.sub.N of a clostridial neurotoxin of a serotype other than botulinum neurotoxin type A, the nucleic acid sequence (SEQ ID NO: 23) coding for the polypeptide LH.sub.107 /B (SEQ ID NO: 24) was inserted into the commercially available plasmid pET28a (Novogen, Madison, Wis., USA). The nucleic acid was expressed in E. coli BL21 (DE3) (New England BioLabs, Beverly, Mass., USA) as a fusion protein with a N-terminal T7 fusion peptide, under IPTG induction at 1 mM for 90 minutes at 37.degree. C. Cultures were harvested and recombinant protein extracted as described previously for LH.sub.423 /A.

Detailed Description Text (44):

Recombinant protein was recovered and purified from bacterial paste lysates by immunoaffinity adsorption to an immobilised anti-T7 peptide monoclonal antibody

using a T7 tag purification kit (New England bioLabs, Beverley, Mass., USA). Purified recombinant protein was analysed by gradient (4-20%) denaturing SDS-polyacrylamide gel electrophoresis (Novex, San Diego, Calif., USA) and western blotting using polyclonal anti-botulinum neurotoxin type antiserum or anti-T7 antiserum. Western blotting reagents were from Novex, immunostained proteins were visualised using the Enhanced Chemi-Luminescence system (ECL) from Amersham. The expression of an anti-T7 antibody and anti-botulinum neurotoxin type B antiserum reactive recombinant product is demonstrated in FIG. 13.

Detailed Description Paragraph Table (2):

TABLE 1 Neutralisation of botulinum neurotoxin in mice by guinea pig anti-GST-.sub.2 .sub.2 LH.sub.423 /A antiserum. Botulinum Toxin/mouse Survivors 0.5 0.005 0.0005 0.5 0.005 5 Control On Day .mu.g .mu.g .mu.g ng ng pg (no toxin) 1 0 4 4 4 4 4 2 -- 4 4 4 4 4 3 -- 4 4 4 4 4 4 4 -- 4 4 4 4 4 4

Detailed Description Paragraph Table (3):

TABLE 2 Neutralisation of botulinum neurotoxin in mice by non-immune guinea pig antiserum. Botulinum Toxin/mouse Survivors 0.5 0.005 0.0005 0.5 0.005 5 Control On Day .mu.g .mu.g .mu.g ng ng pg (no toxin) 1 0 0 0 0 0 2 4 2 -- -- -- -- -- 0 4 3 -- -- -- -- -- 4 4 -- -- -- -- -- 4

Other Reference Publication (2):

Poulain et al, Inhibition of transmitter release by botulinum neurotoxin A, European Journal of Biochemistry, vol. 185, pp. 197-203, 1989.\*

Other Reference Publication (3):

Niemann, H., "Molecular Biology of Clostridial Neurotoxins," In Sourcebook of Bacterial Protein Toxins, Ch. 15, Alouf, J.E. and Freer, J.H., editors, Academic Press Limited, London, pp. 303-348 (1991).

Other Reference Publication (4):

Poulain, B. et al., "Inhibition of transmitter release by botulinum neurotoxin A: Contribution of various fragments to the intoxication process," J. Biochem. 185:197-203 (1989).

Other Reference Publication (5):

Binz, T., et al., "The Complete Sequence of Botulinum Neurotoxin Type A and Comparison with Other Clostridial Neurotoxins," J. Biol. Chem. 265:9153-9158 (Jun. 1990).

Other Reference Publication (6):

Kurazono, H., et al., "Minimal Essential Domains Specifying Toxicity of the Light Chains of Tetanus Toxin and Botulinum Neurotoxin Type A," J. Biol. Chem. 267:14721-14729 (Jul. 1992).

CLAIMS:

1. A non-toxic polypeptide comprising first, second and third domains, wherein (a) said first domain comprises a botulinum toxin light chain and cleaves one or more vesicle or plasma-membrane associated proteins essential to exocytosis, (b) said second domain comprises the first 100 N-terminal amino acids of a botulinum toxin heavy chain and (i) translocates the polypeptide into a cell or (ii) increases the solubility of the polypeptide compared to the solubility of the first domain on its own or (iii) both translocates the polypeptide into a cell and increases the solubility of the polypeptide compared to the solubility of the first domain on its own, (c) said polypeptide is free of clostridial neurotoxin and free of clostridial neurotoxin precursor that can be converted into toxin by proteolytic action, (d) said polypeptide is a single polypeptide, (e) said third domain is a tandem repeat synthetic IgG binding domain derived from domain .beta. of Staphylococcal protein A, and (f) said polypeptide lacks a portion designated H.sub.c of a botulinum toxin

heavy chain.

2. A non-toxin polypeptide comprising first, second and third domains, wherein (a) said first domain comprises a botulinum toxin light chain and cleaves one or more vesicle or plasma-membrane associated proteins essential to exocytosis, (b) said second domain comprises the first 100 N-terminal amino acids of a botulinum toxin heavy chains and (i) translocates the polypeptide into a cell or (ii) increases the solubility of the polypeptide compared to the solubility of the first domain on its own or (iii) both translocates the polypeptide into a cell and increases the solubility of the polypeptide compared to the solubility of the first domain on its own, (c) said polypeptide is free of clostridial neurotoxin and free of clostridial neurotoxin precursor that can be converted into toxin by proteolytic action, (d) said polypeptide is a single polypeptide, (e) said third domain is insulin-like growth-factor-1 (IGF-1), and (f) said polypeptide lacks a portion designated H.sub.c of a botulinum toxin heavy chain.

3. A non-toxin polypeptide comprising first and second domains, wherein (a) said first domain is a botulinum toxin type A light chain variant comprising a sequence correspond to amino acids 1-448 of SEQ ID NO:2 wherein three amino acid residues have been altered compared to that sequence, namely at residue 2 a glutamate, at residue 26 a lysine and at residue 27 a tyrosine which first domain cleaves one or more vesicle or plasma-membrane associated proteins essential to exocytosis, (b) said second domain comprises the first 100 N-terminal amino acids of a botulinum toxin heavy chains and (i) translocates the polypeptide into a cell or (ii) increases the solubility of the polypeptide compared to the solubility of the first domain on its own or (iii) both translocates the polypeptide into a cell and increases the solubility of the polypeptide compared to the solubility of the first domain on its own, (c) said polypeptide is free of clostridial neurotoxin and free of clostridial neurotoxin precursor that can be converted into toxin by proteolytic action, (d) said polypeptide is a single polypeptide, (e) one or both of (i) the toxin light chain or fragment or variant of toxin light chain and (ii) the portion of the toxin heavy chain are of botulinum toxin type A, and (f) said polypeptide lacks a portion designated H.sub.c of a botulinum toxin heavy chain.

4. A non-toxin polypeptide comprising first and second domains, wherein (a) first domain comprises a botulinum toxin light chain and cleaves one or more vesicle or plasma-membrane associated proteins essential to exocytosis, (b) said second domain comprises the first 100 N-terminal amino acids of a botulinum toxin heavy chain and (i) translocates the polypeptide into a cell or (ii) increases the solubility of the the polypeptide compared to the solubility of the first domain on its own or (iii) both translocates the polypeptide into a cell and increases the solubility of the polypeptide compared to the solubility of the first domain on its own, (c) said polypeptide is free of clostridial neurotoxin and free of clostridial neurotoxin precursor that can be converted into toxin by proteolytic action, (d) said polypeptide is a single polypeptide, and (e) said polypeptide lacks a portion designated H.sub.c of a botulinum toxin heavy chain.

5. A non-toxin polypeptide comprising first and second domains, wherein (a) said first domain comprises a botulinum toxin light chain and cleaves one or more vesicle vesicle or plasma-membrane associated proteins essential to exocytosis, (b) said second domain comprises the first 100 N-terminal amino acids of a botulinum toxin heavy chain and (i) translocates the polypeptide into a cell or (ii) increases the solubility of the polypeptide compared to the solubility of the first domain on its own or (iii) both translocates the polypeptide into a cell and increases the solubility of the polypeptide compared to the solubility of the first domain on its own, (c) said polypeptide is free of clostridial neurotoxin and free of clostridial neurotoxin precursor that can be converted into toxin by proteolytic action, (d) said polypeptide is a single polypeptide, (e) said second domain comprises a portion designated H.sub.N of the botulinum toxin heavy chain which consists of the 423 N-terminal amino acids of a botulinum toxin type A heavy chain, and (f) said

polypeptide lacks a portion designated H.sub.c of a botulinum toxin heavy chain.

6. A polypeptide according to claim 5 wherein the first domain comprises a botulinum toxin type A light chain.

7. A polypeptide according to claim 5 wherein said first domain is a botulinum toxin type A light chain variant which comprises a sequence corresponding to amino acids 1-448 of SEQ ID NO:2 having at least three amino acid residues which are altered compared to that sequence, namely at residue 2 a glutamate, residue 26 a lysine and residue 27 a tyrosine, and wherein said polypeptide contains 423 N-terminal amino acids of a botulinum toxin type A heavy chain.

8. A polypeptide comprising a botulinum toxin light chain and a botulinum toxin heavy chain lacking a C-terminal part of the botulinum toxin heavy chain designated H.sub.c wherein said botulinum toxin heavy chain is not capable of binding to cell surface receptors.

9. A polypeptide according to claim 8 wherein said heavy chain lacks amino acid residues 872 -1296 of botulinum toxin A.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Claims	KWC	Draw. De
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☐ 7. Document ID: US 6451593 B1

L5: Entry 7 of 19

File: USPT

Sep 17, 2002

DOCUMENT-IDENTIFIER: US 6451593 B1

**\*\* See image for Certificate of Correction \*\***

TITLE: Design principle for construction of expression constructs for gene therapy

Detailed Description Text (57):

Examples of genetic vaccination methods, procedures, materials, and/or apparatus, which might possibly be used in conjunction with at least one embodiment of the present invention may be found in the following U.S. Patents, which U.S. patents may also contain the full-names of abbreviations found herein, namely, U.S. Patent Nos: U.S. Pat. No. 5,889,038, entitled "Methods and products for treating diarrhea and scours: use of clotrimazole and related aromatic compounds"; U.S. Pat. No. 5,880,103, entitled "Immunomodulatory peptides"; U.S. Pat. No. 5,869,058, entitled "Peptides used as carriers in immunogenic constructs suitable for development of synthetic vaccines"; U.S. Pat. No. 5,866,553, entitled "Polynucleotide vaccine for papillomavirus"; U.S. Pat. No. 5,861,397, entitled "Piperazine based cytofectins"; U.S. Pat. No. 5,861,290, entitled "Methods and polynucleotide constructs for treating host cells for infection or hyperproliferative disorders"; U.S. Pat. No. 5,859,324, entitled "Hypersensitive response induced resistance in plants"; U.S. Pat. No. 5,846,961, entitled "Multi-faceted method to repress reproduction of latent viruses in humans and animals"; U.S. Pat. No. 5,840,707 entitled "Stabilizing and delivery means of biological molecules"; U.S. Pat. No. 5,837,511, entitled "Non-group C adenoviral vectors"; U.S. Pat. No. 5,837,510, entitled "Methods and polynucleotide constructs for treating host cells for infection or hyperproliferative disorders"; U.S. Pat. No. 5,837,269, entitled "Vaccine compositions and method for enhancing an immune response"; U.S. Pat. No. 5,830,876, entitled "Genetic immunization"; U.S. Pat. No. 5,824,313, entitled "Vaccine compositions and method for induction of mucosal immune response via systemic

vaccination"; U.S. Pat. No. 5,817,637, entitled "Genetic immunization"; U.S. Pat. No. 5,780,448, entitled "DNA-based vaccination of fish"; U.S. Pat. No. 5,776,889, entitled "Hypersensitive response induced resistance in plants"; U.S. Pat. No. 5,738,852, entitled "Methods of enhancing antigen-specific T cell responses"; U.S. Pat. No. 5,736,524, entitled "Polynucleotide tuberculosis vaccine"; U.S. Pat. No. 5,891,432, entitled "Membrane-bound cytokine compositions comprising GM.dbd.CSF and methods of modulating an immune response using same"; U.S. Pat. No. 5,889,156, entitled "TNF deletion muteins"; U.S. Pat. No. 5,888,814, entitled "Recombinant host cells encoding TNF proteins"; U.S. Pat. No. 5,888,502, entitled "Recombinant retroviruses"; U.S. Pat. No. 5,882,640, entitled "Treatment of hyperallergenic response with oral interferon"; U.S. Pat. No. 5,879,675, entitled "Compositions and methods for vaccines comprising .alpha.-galactosyl epitopes"; U.S. Pat. No. 5,874,077, entitled "Human til cells expressing recombinant TNF prohormone"; U.S. Pat. No. 5,861,164, entitled "Vaccination against diseases resulting from pathogenic pathogenic responses by specific T cell populations"; U.S. Pat. No. 5,853,765, entitled "Anti-cholesterolemic egg, vaccine and method for production, and use"; U.S. Pat. No. 5,849,586, entitled "Infective protein delivery system"; U.S. Pat. No. 5,846,526, entitled "Treatment of autoimmune disorders with oral interferon"; U.S. Pat. No. 5,837,246, entitled "Vaccination and methods against diseases resulting from pathogenic responses by specific T cell populations"; U.S. Pat. No. 5,830,458, entitled "Method for destroying a diseased human cell"; U.S. Pat. No. 5,830,456, entitled "Treatment of viral disease with oral interferon-.alpha."; U.S. Pat. No. 5,824,300, entitled "Treatment of neoplastic disease with oral interferon"; U.S. Pat. No. 5,817,307, entitled "Treatment of bacterial infection with oral interferon-.alpha."; U.S. Pat. No. 5,804,191, entitled "Sperm as immunogen immunogen carriers"; U.S. Pat. No. 5,804,187, entitled "Modified antibodies with human milk fat globule specificity"; U.S. Pat. No. 5,780,304, entitled "Diagnosis and treatment of insulin dependent diabetes mellitus using heat shock protein determinants"; U.S. Pat. No. 5,776,459, entitled "TCR V beta 5 peptides"; U.S. Pat. No. 5,766,625, entitled "Artificial viral envelopes"; U.S. Pat. No. 5,759,535, entitled "Immunotherapeutic strategies for the treatment of cancer"; U.S. Pat. No. 5,753,262, entitled "Cationic lipid acid salt of 3beta[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol and halogenated solvent-free preliposomal lyophilate thereof"; U.S. Pat. No. 5,753,258, entitled "Artificial viral envelopes"; U.S. Pat. No. 5,736,139, entitled "Treatment of Clostridium difficile induced disease"; U.S. Pat. No. 5,728,385, entitled "Method and composition for an early vaccine to protect against both common infectious diseases and chronic immune mediated disorders or their sequelae"; U.S. Pat. No. 5,723,283, entitled "Method and composition for an early vaccine to protect against both common infectious diseases and chronic immune mediated disorders or their sequelae"; and these patents are hereby incorporated by reference, as if set forth in their entirety herein.

## CLAIMS:

11. A microprojectile for ballistic transfer of a DNA construct into cells, wherein the microprojectile comprises an attached DNA construct, and said DNA construct is selected from the group consisting of: a) a circular strand of DNA which forms a dumbbell comprising a first complementary sequence, a first non-complementary sequence, a second complementary sequence, and a second non-complementary sequence, wherein first and second complementary sequences pair to form a duplex comprising a) a promoter sequence, b) a coding sequence, and c) a polyadenylation or other RNA sequence stabilizing sequence, and wherein first and second non-complementary sequences form single-strand loops; b) a circular strand of DNA which forms a dumbbell comprising a first complementary sequence, a first non-complementary sequence, a second complementary sequence, and a second non-complementary sequence, wherein first and second complementary sequences pair to form a duplex comprising a) a promoter sequence, b) a coding sequence, and c) a polyadenylation sequence or other RNA stabilizing sequence; wherein first and second non-complementary sequences form single-strand loops comprising three to seven nucleotides of which

one or several are covalently modified by carboxy-, amine-, thiole, or aldehyde functional groups; c) a circular strand of DNA which forms a dumbbell comprising a first complementary sequence, a first non-complementary sequence, a second complementary sequence, and a second non-complementary sequence, wherein first and second complementary sequences pair to form a duplex comprising a) a promoter sequence, b) a coding sequence, and c) a polyadenylation sequence or other RNA stabilizing sequence, and wherein first and second non-complementary sequences form single-strand loops comprising three to seven nucleotides of which one or several are covalently modified by carboxy-, amine-, thiole, or aldehyde functional groups which in turn are linked to one or more peptides that direct DNA construct transport across a cell's endosome or into its nucleus. d) a circular strand of DNA which forms a dumbbell comprising a first complementary sequence, a first non-complementary sequence, a second complementary sequence, and a second non-complementary sequence, wherein first and second complementary sequences pair to form a duplex comprising a) a promoter sequence comprising a 7SK promoter or a CMV promoter, b) a coding sequence, and c) a polyadenylation sequence or other RNA stabilizing sequence, and wherein first and second non-complementary sequences form single-strand loops; e) a circular strand of DNA which forms a dumbbell comprising a first complementary sequence, a first non-complementary sequence, a second complementary sequence, and a second non-complementary sequence, wherein first and second complementary sequences pair to form a duplex comprising a promoter sequence operably linked to a coding sequence for interleukin-7, interleukin-12 or at least one of its constitutive subunits, gm-csf, p16 or p53 protein or fragments thereof, or peptide fragments of mutated ki-ras, mutated p53 or bcr-abl translocation product with a length of between 10 and 100 amino acids; a polyadenylation sequence or other RNA stabilizing sequence, and wherein first and second non-complementary sequences form single-strand loops; f) a circular strand of DNA which forms a dumbbell comprising a first complementary sequence, a first non-complementary sequence, a second complementary sequence, and a second non-complementary sequence, wherein first and second complementary sequences pair to form a duplex comprising a promoter sequence, a coding sequence for interleukin-7, interleukin-12 or at least one of its constitutive subunits, gm-csf, p16 or p53 protein or fragments thereof, or peptide fragments of mutated ki-ras, mutated p53 or bcr-abl translocation product with a length of between 10 and 100 amino acids; a polyadenylation sequence or other RNA stabilizing sequence; and wherein first and second non-complementary sequences form single-strand loops comprising three to seven nucleotides, in which one or several of said nucleotides are covalently modified by carboxylic-, amine-, thiole or aldehyde functional groups; g) a circular strand of DNA which forms a dumbbell comprising a first complementary sequence, a first non-complementary sequence, a second complementary sequence, and a second non-complementary sequence, wherein first and second complementary sequences pair to form a duplex comprising a promoter sequence, a coding sequence for interleukin-7, interleukin-12 or at least one of its constitutive subunits, gm-csf, p16 or p53 protein or fragments thereof, or peptide fragments of mutated ki-ras, mutated p53 or bcr-abl translocation product with a length of between 10 and 100 amino acids; a polyadenylation sequence or other RNA stabilizing sequence; and wherein first and second non-complementary sequences form single-strand loops comprising three to seven nucleotides, in which one or several of said nucleotides are covalently modified by carboxylic-, amine-, thiole or aldehyde functional groups which in turn are linked to one or more peptides that direct DNA construct transport across a cell's endosome or into its nucleus; h) a circular strand of DNA which forms a dumbbell comprising a first complementary sequence, a first non-complementary sequence, a second complementary sequence, and a second non-complementary sequence, wherein first and second complementary sequences pair to form a duplex comprising a promoter sequence comprising a 7SK promoter or a CMV promoter, a coding sequence for interleukin-7, interleukin-12 or at least one of its constitutive subunits, gm-csf, p16 or p53 protein or fragments thereof, or peptide fragments of mutated ki-ras, mutated p53 or bcr-abl translocation product with a length of between 10 and 100 amino acids; a polyadenylation sequence or other RNA stabilizing sequence; and wherein first and second non-complementary sequences form single-strand loops.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Keywords	Claims	KMC	Draw D
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8. Document ID: US 6416959 B1

L5: Entry 8 of 19

File: USPT

Jul 9, 2002

DOCUMENT-IDENTIFIER: US 6416959 B1

TITLE: System for cell-based screening

Detailed Description Text (357):

Another example of a protease class for which this biosensor can be constructed to report activity is zinc metalloproteases. Two specific examples of this class are the biological toxins derived from Clostridial species (C. botulinum and C. tetani) and Bacillus anthracis. (Herrerros et al. In The Comprehensive Sourcebook of Bacterial Protein Toxins. J. E. Alouf and J. H. Freer, Eds. 2.sup.nd edition, San Diego, Academic Press, 1999; pp 202-228.) These bacteria express and secrete zinc metalloproteases that enter eukaryotic cells and specifically cleave distinct target proteins. For example, the anthrax protease from Bacillus anthracis is delivered into the cytoplasm of target cells via an accessory pore-forming protein, where its proteolytic activity inactivates the MAP-kinase signaling cascade through cleavage of mitogen activated protein kinase kinases 1 or 2 (MEK1 or MEK2). (Leppla, S. A. In The Comprehensive Sourcebook of Bacterial Protein Toxins. J. E. Alouf and J. H. Freer, Eds. 2.sup.nd edition, San Diego, Academic Press, 1999; pp243-263.) The toxin biosensors described here take advantage of the natural subcellular localization of these and other target proteins to achieve reactant targeting. Upon cleavage, the signal (with or without a product target sequence) is separated from the reactant to create a high-content biosensor.

Detailed Description Text (476):

5. Tetanus/Botulinum Biosensor with Trans-membrane Targeting Domain

Detailed Description Text (477):

In an alternative embodiment, a trans-membrane targeting sequence is used to tether the reactant to cytoplasmic vesicles, and an alternative protease recognition site is used. The tetanus/botulinum biosensor (SEQ ID NOS:27-28 (cellubrevin); 29-30 (synaptobrevin) consists of an NLS (SEQ ID NO:128) (FIG. 29C), Fret25 signal domain (SEQ ID NO:52) (FIG. 29A), a tetanus or botulinum zinc metalloprotease recognition site from cellubrevin (SEQ ID NO:106) (FIG. 29B) (McMahon et al., Nature 364:346-349, 1993; Martin et al., J. Cell Biol., in press) or synaptobrevin (SEQ ID NO:108) (FIG. 29B) (GenBank Accession #U64520), and a trans-membrane sequence from cellubrevin (SEQ ID NO:146) (FIG. 29C) or synaptobrevin (SEQ ID NO:144) (FIG. 29C) at the 3'-end which tethers the biosensor to cellular vesicles. The N-terminus of each protein is oriented towards the cytoplasm. In the intact biosensor, GFP is tethered to the vesicles. Upon cleavage by the tetanus or botulinum zinc metalloprotease, GFP will no longer be associated with the vesicle and is free to diffuse throughout the cytoplasm and the nucleus.

Detailed Description Text (546):

ADP-ribosylating toxins--These toxins include Pseudomonas toxin A, diphtheria toxin, botulinum toxin, pertussis toxin, and cholera toxin. For example, C. botulinum C2 toxin induces the ADP-ribosylation of Arg177 in the cytoskeletal protein actin, thus altering its assembly properties. Besides the construction of a classifier

assay to measure actin-cytoskeleton regulation, an identifier assay can be constructed to detect the specific actin ADP-ribosylation. Because the ADP-ribosylation induces a conformational change that no longer permits the modified actin to polymerize, this conformational change can be detected intracellularly in several possible ways using luminescent reagents. For example, actin can be luminescently labeled using a fluorescent reagent with an appropriate excited state lifetime that allows for the measurement of the rotational diffusion of the intracellular actin using steady state fluorescence anisotropy. That is, toxin-modified actin will no longer be able to assemble into rigid filaments and will therefore produce only luminescent signals with relatively low anisotropy, which can be readily measured with an imaging system. In another embodiment, actin can be labeled with a polarity-sensitive fluorescent reagent that reports changes in actin-actin-conformation through spectral shifts of the attached reagent. That is, toxin-treatment will induce a conformational change in intracellular actin such that a ratio of two fluorescence wavelengths will provide a measure of actin ADP-ribosylation.

Detailed Description Text (547):

Cytotoxic phospholipases--Several gram-positive bacterial species produce cytotoxic phospholipases. For example, Clostridium perfringens produces a phospholipase C specific for the cleavage of phosphoinositides. These phosphoinositides (e.g., inositol 1,4,5-trisphosphate) induce the release of calcium ions from intracellular organelles. An assay that can be conducted as either high-content or high-throughput can be constructed to measure the release of calcium ions using fluorescent reagents that have altered spectral properties when complexed with the metal ion. Therefore, a direct consequence of the action of a phospholipase C based toxin can be measured as a change in cellular calcium ion concentration.

Detailed Description Text (551):

A number of such protease biosensors (including FRET biosensors) are disclosed above, such as the caspase biosensors, anthrax, tetanus, Botulinum, and the zinc metalloproteases. FRET is a powerful technique in that small changes in protein conformation, many of which are associated with toxin activity, can not only be measured with high precision in time and space within living cells, but can be measured in a high-throughput mode, as discussed above.

Detailed Description Paragraph Table (21):

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Detailed Description Paragraph Table (22):

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## CLAIMS:

10. The method of any of claims 1-4 wherein the change in the localization, distribution, structure or activity of the first, second, or third luminescent reporter molecules is selected from the group consisting of cytoplasm to nucleus translocation, nucleus or nucleolus to cytoplasm translocation, receptor internalization, mitochondrial membrane potential, loss of signal, the spectral response of the reporter molecule, phosphorylation, intracellular free ion concentration, cell size, cell shape, cytoskeleton organization, metabolic processes, cell motility, cell substrate attachment, cell cycle events, and organellar structure and function.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Annotations	Claims	KMC	Draw. De
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☐ 9. Document ID: US 6312708 B1

L5: Entry 9 of 19

File: USPT

Nov 6, 2001

DOCUMENT-IDENTIFIER: US 6312708 B1

TITLE: Botulinum toxin implantAbstract Text (1):

A controlled release system for multiphasic, in vivo release of therapeutic amounts of botulinum toxin in a human patient over a prolonged period of time. The controlled release system can comprise a plurality of botulinum toxin incorporating polymeric microspheres.

Brief Summary Text (2):

The present invention relates to an implantable drug delivery system. In particular, the present invention relates to an implantable botulinum toxin delivery system.

Brief Summary Text (23):Botulinum ToxinBrief Summary Text (24):

The anaerobic, gram positive bacterium Clostridium botulinum produces a potent polypeptide neurotoxin, botulinum toxin, which causes a neuroparalytic illness in humans and animals referred to as botulism. The spores of Clostridium botulinum are found in soil and can grow in improperly sterilized and sealed food containers of home based canneries, which are the cause of many of the cases of botulism. The effects of botulism typically appear 18 to 36 hours after eating the foodstuffs infected with a Clostridium botulinum culture or spores. The botulinum toxin can apparently pass unattenuated through the lining of the gut and attack peripheral motor neurons. Symptoms of botulinum toxin intoxication can include nausea, difficulty walking and swallowing, and can progress to paralysis of respiratory muscles, cardiac failure and death.

Brief Summary Text (25):

Botulinum toxin type A is the most lethal natural biological agent known to man. About 50 picograms of a commercially available botulinum toxin type A (available from Allergan, Inc., Irvine, Calif. under the tradename BOTOX.RTM. (purified neurotoxin complex) in 100 unit vials) is a LD.sub.50 in mice (i.e. 1 unit). Thus, one unit of BOTOX.RTM. contains about 50 picograms (about 56 attomoles) of botulinum toxin type A complex. Interestingly, on a molar basis, botulinum toxin type A is about 1.8 billion times more lethal than diphtheria, about 600 million times more lethal than sodium cyanide, about 30 million times more lethal than cobra toxin and about 12 million times more lethal than cholera. Singh, Critical Aspects of Bacterial Protein Toxins, pages 63-84 (chapter 4) of Natural Toxins II, edited by B. R. Singh et al., Plenum Press, New York (1996) (where the stated LD.sub.50 of botulinum toxin type A of 0.3 ng equals 1 U is corrected for the fact that about 0.05 ng of BOTOX.RTM. equals 1 unit). One unit (U) of botulinum toxin is defined as the LD.sub.50 upon intraperitoneal injection into female Swiss Webster mice weighing 18 to 20 grams each.

Brief Summary Text (26):

Neurotransmitters are packaged in synaptic vesicles within the cytoplasm of neurons and are then transported to the inner plasma membrane where the vesicles dock and fuse with the plasma membrane. Recent studies of nerve cells employing clostridial

neurotoxins as probes of membrane fusion have revealed that fusion of synaptic vesicles with the cell membrane in nerve cells depends upon the presence of specific proteins that are associated with either the vesicle or the target membrane. These proteins have been termed SNAREs. A protein alternatively termed synaptobrevin or VAMP (vesicle-associated membrane protein) is a vesicle-associated SNARE (v-SNARE). There are at least two isoforms of synaptobrevin; these two isoforms are differentially expressed in the mammalian central nervous system, and are selectively associated with synaptic vesicles in neurons and secretory organelles in neuroendocrine cells. The target membrane-associated SNAREs (t-SNAREs) include syntaxin and SNAP-25. Following docking, the VAMP protein forms a core complex with syntaxin and SNAP-25; the formation of the core complex appears to be an essential step to membrane fusion. See Neimann et al., Trends in Cell Biol. 4:179-185:1994.

Brief Summary Text (27):

Seven generally immunologically distinct botulinum neurotoxins have been characterized, these being respectively botulinum neurotoxin serotypes A, B, C.sub.1, D, E, F and G each of which is distinguished by neutralization with type-specific antibodies. The different serotypes of botulinum toxin vary in the animal species that they affect and in the severity and duration of the paralysis they evoke. For example, it has been determined that botulinum toxin type A is 500 times more potent, as measured by the rate of paralysis produced in the rat, than is botulinum toxin type B. Additionally, botulinum toxin type B has been determined to be non-toxic in primates at a dose of 480 U/kg which is about 12 times the primate LD.sub.50 for botulinum toxin type A. Botulinum toxin apparently binds with high affinity to cholinergic motor neurons, is translocated into the neuron and blocks the release of acetylcholine.

Brief Summary Text (28):

Regardless of serotype, the molecular mechanism of toxin intoxication appears to be similar and to involve at least three steps or stages. In the first step of the process, the toxin binds to the presynaptic membrane of the target neuron through a specific interaction between the heavy chain, H chain, and a cell surface receptor; the receptor is thought to be different for each type of botulinum toxin and for tetanus toxin. The carboxyl end segment of the H chain, H.sub.C, appears to be important for targeting of the toxin to the cell surface.

Brief Summary Text (30):

The last step of the mechanism of botulinum toxin activity appears to involve reduction of the disulfide bond joining the heavy chain, H chain, and the light chain, L chain. The entire toxic activity of botulinum and tetanus toxins is contained in the L chain of the holotoxin; the L chain is a zinc (Zn++) endopeptidase which selectively cleaves proteins essential for recognition and docking of neurotransmitter-containing vesicles with the cytoplasmic surface of the plasma membrane, and fusion of the vesicles with the plasma membrane. Tetanus neurotoxin, and botulinum toxins B, D, F, and G cause degradation of synaptobrevin (also called vesicle-associated membrane protein (VAMP)), a synaptosomal membrane protein. Most of the VAMP present at the cytoplasmic surface of the synaptic vesicle is removed as a result of any one of these cleavage events. Serotype A and E cleave SNAP-25. Serotype C.sub.1 was originally thought to cleave syntaxin, but was found to cleave syntaxin and SNAP-25. Each toxin specifically cleaves a different bond (except tetanus and type B which cleave the same bond).

Brief Summary Text (31):

Botulinum toxins have been used in clinical settings for the treatment of neuromuscular disorders characterized by hyperactive skeletal muscles. Botulinum toxin type A was approved by the U.S. Food and Drug Administration in 1989 for the treatment of blepharospasm, strabismus and hemifacial spasm. Non-type A botulinum toxin serotypes apparently have a lower potency and/or a shorter duration of activity as compared to botulinum toxin type A. Clinical effects of peripheral

intramuscular botulinum toxin type A are usually seen within one week of injection. The typical duration of symptomatic relief from a single intramuscular injection of botulinum toxin type A averages about three months.

Brief Summary Text (32):

Although all the botulinum toxins serotypes apparently inhibit release of the neurotransmitter acetylcholine at the neuromuscular junction, they do so by affecting different neurosecretory proteins and/or cleaving these proteins at different sites. For example, botulinum types A and E both cleave the 25 kiloDalton (kD) synaptosomal associated protein (SNAP-25), but they target different amino acid sequences within this protein. Botulinum toxin types B, D, F and G act on vesicle-associated protein (VAMP, also called synaptobrevin), with each serotype cleaving the protein at a different site. Finally, botulinum toxin type C.sub.1 has been shown to cleave both syntaxin and SNAP-25. These differences in mechanism of action may affect the relative potency and/or duration of action of the various botulinum toxin serotypes. Apparently, a substrate for a botulinum toxin can be found in a variety of different cell types. See e.g. Biochem, J 1;339 (pt 1):159-65:1999, and Mov Disord, 10(3): 376:1995 (pancreatic islet B cells contain at least SNAP-25 and synaptobrevin).

Brief Summary Text (33):

The molecular weight of the botulinum toxin protein molecule, for all seven of the known botulinum toxin serotypes, is about 150 kD. Interestingly, the botulinum toxins are released by Clostridial bacterium as complexes comprising the 150 kD botulinum toxin protein molecule along with associated non-toxin proteins. Thus, the botulinum toxin type A complex can be produced by Clostridial bacterium as 900 kD, 500 kD and 300 kD forms. Botulinum toxin types B and C.sub.1 is apparently produced as only a 700 kD or 500 kD complex. Botulinum toxin type D is produced as both 300 kD and 500 kD complexes. Finally, botulinum toxin types E and F are produced as only approximately 300 kD complexes. The complexes (i.e. molecular weight greater than about 150 kD) are believed to contain a non-toxin hemagglutinin protein and a non-toxin and non-toxic nonhemagglutinin protein. These two non-toxin proteins (which along with the botulinum toxin molecule comprise the relevant neurotoxin complex) may act to provide stability against denaturation to the botulinum toxin molecule and protection against digestive acids when toxin is ingested. Additionally, it is possible that the larger (greater than about 150 kD molecular weight) botulinum toxin complexes may result in a slower rate of diffusion of the botulinum toxin away from a site of intramuscular injection of a botulinum toxin complex.

Brief Summary Text (34):

In vitro studies have indicated that botulinum toxin inhibits potassium cation induced release of both acetylcholine and norepinephrine from primary cell cultures of brainstem tissue. Additionally, it has been reported that botulinum toxin inhibits the evoked release of both glycine and glutamate in primary cultures of spinal cord neurons and that in brain synaptosome preparations botulinum toxin inhibits the release of each of the neurotransmitters acetylcholine, dopamine, norepinephrine (Habermann E., et al., Tetanus Toxin and Botulinum A and C Neurotoxins Inhibit Noradrenaline Release From Cultured Mouse Brain, J Neurochem 51 (2);522-527:1988) CGRP, substance P and glutamate (Sanchez-Prieto, J., et al., Botulinum Toxin A Blocks Glutamate Exocytosis From Guinea Pig Cerebral Cortical Synaptosomes, Eur J. Biochem 165;675-681:1987. Thus, when adequate concentrations are used, stimulus-evoked release of most neurotransmitters is blocked by botulinum toxin. See e.g. Pearce, L. B., Pharmacologic Characterization of Botulinum Toxin For Basic Science and Medicine, Toxicon 35(9);1373-1412 at 1393 (1997); Bigalke H., et al., Botulinum A Neurotoxin Inhibits Non-Cholinergic Synaptic Transmission in Mouse Spinal Cord Neurons in Culture, Brain Research 360;318-324:1985; Habermann E., Inhibition by Tetanus and Botulinum A Toxin of the Release of [.sup.3 H] Noradrenaline and [.sup.3 H]GABA From Rat Brain Homogenate, Experientia 44;224-226:1988, Bigalke H., et al., Tetanus Toxin and Botulinum A Toxin Inhibit Release

and Uptake of Various Transmitters, as Studied with Particulate Preparations From Rat Brain and Spinal Cord, Naunyn-Schmiedeberg's Arch Pharmacol 316;244-251:1981, and; Jankovic J. et al., Therapy With Botulinum Toxin, Marcel Dekker, Inc., (1994), page 5.

Brief Summary Text (35):

Botulinum toxin type A can be obtained by establishing and growing cultures of Clostridium botulinum in a fermenter and then harvesting and purifying the fermented fermented mixture in accordance with known procedures. All the botulinum toxin serotypes are initially synthesized as inactive single chain proteins which must be cleaved or nicked by proteases to become neuroactive. The bacterial strains that make botulinum toxin serotypes A and G possess endogenous proteases and serotypes A and G can therefore be recovered from bacterial cultures in predominantly their active form. In contrast, botulinum toxin serotypes C.sub.1, D and E are synthesized synthesized by nonproteolytic strains and are therefore typically unactivated when recovered from culture. Serotypes B and F are produced by both proteolytic and nonproteolytic strains and therefore can be recovered in either the active or inactive form. However, even the proteolytic strains that produce, for example, the botulinum toxin type B serotype only cleave a portion of the toxin produced. The exact proportion of nicked to unnicked molecules depends on the length of incubation and the temperature of the culture. Therefore, a certain percentage of any preparation of, for example, the botulinum toxin type B toxin is likely to be inactive, possibly accounting for the known significantly lower potency of botulinum toxin type B as compared to botulinum toxin type A. The presence of inactive botulinum toxin molecules in a clinical preparation will contribute to the overall protein load of the preparation, which has been linked to increased antigenicity, without contributing to its clinical efficacy. Additionally, it is known that botulinum toxin type B has, upon intramuscular injection, a shorter duration of activity and is also less potent than botulinum toxin type A at the same dose level.

Brief Summary Text (36):

High quality crystalline botulinum toxin type A can be produced from the Hall A strain of Clostridium botulinum with characteristics of .gtoreq.3.times.10.sup.7 U/mg, an A.sub.260 /A.sub.278 of less than 0.60 and a distinct pattern of banding on gel electrophoresis. The known Shantz process can be used to obtain crystalline botulinum toxin type A, as set forth in Shantz, E. J., et al, Properties and Use of Botulinum Toxin and Other Microbial Neurotoxins in Medicine, Microbiol Rev. 56;80-99:1992. Generally, the botulinum toxin type A complex can be isolated and purified from an anaerobic fermentation by cultivating Clostridium botulinum type A in a suitable medium. The known process can also be used, upon separation out of the non-toxin proteins, to obtain pure botulinum toxins, such as for example: purified botulinum toxin type A with an approximately 150 kD molecular weight with a specific specific potency of 1-2.times.10.sup.8 LD.sub.50 U/mg or greater; purified botulinum toxin type B with an approximately 156 kD molecular weight with a specific specific potency of 1-2.times.10.sup.8 LD.sub.50 U/mg or greater, and; purified botulinum toxin type F with an approximately 155 kD molecular weight with a specific specific potency of 1-2.times.10.sup.7 LD.sub.50 U/mg or greater.

Brief Summary Text (37):

Botulinum toxins and/or botulinum toxin complexes can be obtained from various sources, including List Biological Laboratories, Inc., Campbell, Calif.; the Centre for Applied Microbiology and Research, Porton Down, U.K.; Wako (Osaka, Japan), Metabiologics (Madison, Wis.) as well as from Sigma Chemicals of St. Louis, Mo.

Brief Summary Text (38):

Pure botulinum toxin is so labile that it is generally not used to prepare a pharmaceutical composition. Furthermore, the botulinum toxin complexes, such as the toxin type A complex are also extremely susceptible to denaturation due to surface denaturation, heat, and alkaline conditions. Inactivated toxin forms toxoid

proteins which can be immunogenic. The resulting antibodies can render a patient refractory to toxin injection.

Brief Summary Text (39):

As with enzymes generally, the biological activities of the botulinum toxins (which are intracellular peptidases) are dependent, at least in part, upon their three dimensional conformation. Thus, botulinum toxin type A is detoxified by heat, various chemicals surface stretching and surface drying. Additionally, it is known that dilution of the toxin complex obtained by the known culturing, fermentation and purification to the much, much lower toxin concentrations used for pharmaceutical composition formulation results in rapid detoxification of the toxin unless a suitable stabilizing agent is present. Dilution of the toxin from milligram quantities to a solution containing nanograms per milliliter presents significant difficulties because of the rapid loss of specific toxicity upon such great dilution. Additionally, the toxin may be used months or years after the toxin containing pharmaceutical composition is formulated. Significantly, it is known that the toxin can be stabilized during the manufacture and compounding processes as well as during storage by use of a stabilizing agent such as albumin and gelatin.

Brief Summary Text (40):

The commercially available botulinum toxin sold under the trademark BOTOX.RTM. (available from Allergan, Inc., of Irvine, Calif.). BOTOX.RTM. consists of a freeze-dried, purified botulinum toxin type A complex, albumin and sodium chloride packaged in sterile, vacuum-dried form. The botulinum toxin type A is made from a culture of the Hall strain of Clostridium botulinum grown in a medium containing N-Z Z amine and yeast extract. The botulinum toxin type A complex is purified from the culture solution by a series of acid precipitations to a crystalline complex consisting of the active high molecular weight toxin protein and an associated hemagglutinin protein. The crystalline complex is re-dissolved in a solution containing saline and albumin and sterile filtered (0.2 microns) prior to vacuum-drying. The vacuum-dried product is stored in a freezer at or below -5.degree. C. BOTOX.RTM. can be reconstituted with sterile, non-preserved saline prior to intramuscular injection. Each vial of BOTOX.RTM. contains about 100 units (U) of Clostridium botulinum toxin type A purified neurotoxin complex, 0.5 milligrams of human serum albumin and 0.9 milligrams of sodium chloride in a sterile, vacuum-dried form without a preservative.

Brief Summary Text (42):

It has been reported that botulinum toxin type A has been used in various clinical settings, including the following:

Brief Summary Text (55):

It is known that botulinum toxin type A can have an efficacy for up to 12 months (European J. Neurology 6 (Supp 4): S111-S1150:1999), and in some circumstances for as long as 27 months, (The Laryngoscope 109: 1344-1346:1999). However, the usual duration of the paralytic effect of an intramuscular injection of Botox.RTM. is typically about 3 to 4 months.

Brief Summary Text (56):

The success of botulinum toxin type A to treat a variety of clinical conditions has led to interest in other botulinum toxin serotypes. A study of two commercially available botulinum type A preparations (BOTOX.RTM. and Dysport.RTM.) and preparations of botulinum toxins type B and F (both obtained from Wako Chemicals, Japan) has been carried out to determine local muscle weakening efficacy, safety and antigenic potential. Botulinum toxin preparations were injected into the head of of the right gastrocnemius muscle (0.5 to 200.0 units/kg) and muscle weakness was assessed using the mouse digit abduction scoring assay (DAS). ED.sub.50 values were calculated from dose response curves. Additional mice were given intramuscular injections to determine LD.sub.50 doses. The therapeutic index was calculated as

LD.sub.50 /ED.sub.50. Separate groups of mice received hind limb injections of BOTOX.RTM. (5.0 to 10.0 units/kg) or botulinum toxin type B (50.0 to 400.0 units/kg), and were tested for muscle weakness and increased water consumption, the later being a putative model for dry mouth. Antigenic potential was assessed by monthly intramuscular injections in rabbits (1.5 or 6.5 ng/kg for botulinum toxin type B or 0.15 ng/kg for BOTOX.RTM.). Peak muscle weakness and duration were dose related for all serotypes. DAS ED.sub.50 values (units/kg) were as follows: BOTOX.RTM.: 6.7, Dysport.RTM.: 24.7, botulinum toxin type B: 27.0 to 244.0, botulinum toxin type F: 4.3. BOTOX.RTM. had a longer duration of action than botulinum toxin type B or botulinum toxin type F. Therapeutic index values were as follows: BOTOX.RTM.: 10.5, Dysport.RTM.: 6.3, botulinum toxin type B: 3.2. Water consumption was greater in mice injected with botulinum toxin type B than with BOTOX.RTM., although botulinum toxin type B was less effective at weakening muscles. After four months of injections 2 of 4 (where treated with 1.5 ng/kg) and 4 of 4 (where treated with 6.5 ng/kg) rabbits developed antibodies against botulinum toxin type B. In a separate study, 0 of 9 BOTOX.RTM. treated rabbits demonstrated antibodies against botulinum toxin type A. DAS results indicate relative peak potencies of botulinum toxin type A being equal to botulinum toxin type F, and botulinum toxin type F being greater than botulinum toxin type B. With regard to duration of effect, botulinum toxin type A was greater than botulinum toxin type B, and botulinum toxin type B duration of effect was greater than botulinum toxin type F. As shown by the therapeutic index values, the two commercial commercial preparations of botulinum toxin type A (BOTOX.RTM. and Dysport.RTM.) are different. The increased water consumption behavior observed following hind limb injection of botulinum toxin type B indicates that clinically significant amounts of of this serotype entered the murine systemic circulation. The results also indicate that in order to achieve efficacy comparable to botulinum toxin type A, it is necessary to increase doses of the other serotypes examined. Increased dosage can comprise safety. Furthermore, in rabbits, type B was more antigenic than was BOTOX.RTM., possibly because of the higher protein load injected to achieve an effective dose of botulinum toxin type B. Eur J Neurol Nov. 6, 1999(Suppl 4):S3-S10. S10.

Brief Summary Text (57):

In addition to having pharmacologic actions at a peripheral location, a botulinum toxin can also exhibit a denervation effect in the central nervous system. Wiegand et al, Naunyn-Schmiedeberg's Arch. Pharmacol. 1976; 292, 161-165, and Habermann, Naunyn-Schmiedeberg's Arch. Pharmacol. 1974; 281, 47-56 reported that botulinum toxin is able to ascend to the spinal area by retrograde transport. As such, a botulinum toxin injected at a peripheral location, for example intramuscularly, can potentially be retrograde transported to the spinal cord.

Brief Summary Text (58):

U.S. Pat. No. 5,989,545 discloses that a modified clostridial neurotoxin or fragment thereof, preferably a botulinum toxin, chemically conjugated or recombinantly fused to a particular targeting moiety can be used to treat pain by administration of the agent to the spinal cord.

Brief Summary Text (59):

At the present time, essentially all therapeutic use of a botulinum toxin is by subcutaneous or intramuscular injection of an aqueous solution of a botulinum toxin type A or B. Typically, a repeat injection must be administered every 2-4 months in order to maintain the therapeutic efficacy of the toxin (i.e. a reduction of muscle spasm at or in the vicinity of the injection site). Each administration of a dose of a botulinum toxin to a patient therefore requires the patient to present himself to his physician at regular intervals. Unfortunately, patients can forget or be unable to attend appointments and physician schedules can make regular, periodic care over a multiyear period difficult to consistently maintain. Additionally, the requirement for 3-6 toxin injections per year on an ongoing basis increases the risk of infection or of misdosing the patient.

Brief Summary Text (64):

Acetylcholine is released from cholinergic neurons when small, clear, intracellular vesicles fuse with the presynaptic neuronal cell membrane. A wide variety of non-neuronal secretory cells, such as, adrenal medulla (as well as the PC12 cell line) and pancreatic islet cells release catecholamines and parathyroid hormone, respectively, from large dense-core vesicles. The PC12 cell line is a clone of rat pheochromocytoma cells extensively used as a tissue culture model for studies of sympathoadrenal development. Botulinum toxin inhibits the release of both types of compounds from both types of cells in vitro, permeabilized (as by electroporation) or by direct injection of the toxin into the denervated cell. Botulinum toxin is also known to block release of the neurotransmitter glutamate from cortical synaptosomes cell cultures.

Brief Summary Text (67):

The tetanus toxin bears many similarities to the botulinum toxins. Thus, both the tetanus toxin and the botulinum toxins are polypeptides made by closely related species of Clostridium (Clostridium tetani and Clostridium botulinum, respectively). Additionally, both the tetanus toxin and the botulinum toxins are dichain proteins composed of a light chain (molecular weight about 50 kD) covalently bound by a single disulfide bond to a heavy chain (molecular weight about 100 kD). Hence, the molecular weight of tetanus toxin and of each of the seven botulinum toxins (non-complexed) is about 150 kD. Furthermore, for both the tetanus toxin and the botulinum toxins, the light chain bears the domain which exhibits intracellular biological (protease) activity, while the heavy chain comprises the receptor binding (immunogenic) and cell membrane translocational domains.

Brief Summary Text (68):

Further, both the tetanus toxin and the botulinum toxins exhibit a high, specific affinity for gangliocide receptors on the surface of presynaptic cholinergic neurons. Receptor mediated endocytosis of tetanus toxin by peripheral cholinergic neurons results in retrograde axonal transport, blocking of the release of inhibitory neurotransmitters from central synapses and a spastic paralysis. Receptor mediated endocytosis of botulinum toxin by peripheral cholinergic neurons results in little if any retrograde transport, inhibition of acetylcholine exocytosis from the intoxicated peripheral motor neurons and a flaccid paralysis.

Brief Summary Text (69):

Finally, the tetanus toxin and the botulinum toxins resemble each other in both biosynthesis and molecular architecture. Thus, there is an overall 34% identity between the protein sequences of tetanus toxin and botulinum toxin type A, and a sequence identity as high as 62% for some functional domains. Binz T. et al., The Complete Sequence of Botulinum Neurotoxin Type A and Comparison with Other Clostridial Neurotoxins, J Biological Chemistry 265(16);9153-9158:1990.

Brief Summary Text (70):

A toxoid is an antigen which can be used to raise antibodies to and thereby vaccinate against the toxin from which the toxoid is derived. Typically, the toxoid comprises the immunogenic fragment of the toxin (i.e. the carboxyl terminal of the heavy chain (designed as H.sub.C) of the tetanus toxin or the botulinum toxins) or a toxin rendered biologically inactive, though still immunogenic, by thermal or chemical (i.e. formalin treatment) denaturation or alteration of the native toxin. Thus, unlike the natural toxin, the toxoid derived from the tetanus or botulinum toxin has been derived of its biological activity, that is its ability to act as an intracellular protease and inhibit neuronal exocytosis of acetylcholine.

Brief Summary Text (77):

Tetanus and botulinum toxoid vaccines have been made by treating the native toxin with formalin. The U.S. Center for Disease Control can supply a pentavalent,

formalin-inactivated toxoid of botulinum toxin types A, B, C, D and E. The pre-exposure immunization schedule calls for subcutaneous administration of the botulinum toxoid vaccine in three dosings at 0, 2 and 12 weeks with a booster at plus 12 months and yearly boosters at yearly intervals thereafter if antibody levels fall.

Brief Summary Text (78):

U.S. Pat. No. 5,980,948 discusses use of polyetherester copolymer microspheres for encapsulation and controlled delivery of a variety of protein drugs, including tetanus and botulinum antitoxins.

Brief Summary Text (79):

U.S. Pat. No. 5,902,565 discusses A controlled or delayed-release preparation comprising microspherical particles comprising a continuous matrix of biodegradable polymer containing discrete, immunogen-containing regions, where the immunogens can be botulinum toxin type C and D toxoids.

Brief Summary Text (80):

What is needed therefore is a biocompatible, pulsatile release, botulinum toxin delivery system by which therapeutic amounts of the botulinum toxin can be locally administered in vivo to a human patient over a prolonged period of time.

Brief Summary Text (82):

The present invention meets this need and provides a biocompatible, pulsatile release, botulinum toxin delivery system by which therapeutic amounts of the botulinum toxin can be locally administered in vivo to a human patient over a prolonged period of time.

Brief Summary Text (83):

The present invention provides a botulinum toxin implant which overcomes the known problems, difficulties and deficiencies associated with repetitive bolus or subcutaneous injection of a botulinum toxin, to treat an affliction such as a movement disorder, including a muscle spasm.

Brief Summary Text (84):

A pulsatile release botulinum toxin delivery system within the scope of the present invention can comprise a carrier material and a botulinum toxin associated with the carrier. The toxin can be associated with the carrier by being mixed with and encapsulated by the carrier to thereby form a pulsatile release botulinum toxin delivery system, that is a botulinum toxin implant. The implant can release therapeutic amounts of the botulinum toxin from the carrier in a plurality of pulses in vivo upon subdermal implantation of the implant system into a human patient. "Subdermal" implantation includes subcutaneous, intramuscular, intraglandular and intracranial sites of implantation.

Brief Summary Text (85):

Preferably, the carrier comprises a plurality of polymeric microspheres (i.e. a polymeric matrix) and substantial amounts of the botulinum toxin has not be transformed into a botulinum toxoid prior to association of the botulinum toxin with the carrier. That is, significant amounts of the botulinum toxin associated with the carrier have a toxicity which is substantially unchanged relative to the toxicity of the botulinum toxin prior to association of the botulinum toxin with the the carrier.

Brief Summary Text (86):

According to the present invention, the botulinum toxin can be released from the carrier over of a period of time of from about 10 days to about 6 years and the carrier is comprised of a substance which is substantially biodegradable.

Brief Summary Text (87):

The botulinum toxin is one of the botulinum toxin types A, B, C.sub.1, D, E, F and G and is preferably botulinum toxin type A. The botulinum toxin can be associated with the carrier in an amount of between about 1 unit and about 50,000 units of the botulinum toxin. Preferably, the quantity of the botulinum toxin associated with the carrier is between about 10 units and about 2,000 units of a botulinum toxin type A. Where the botulinum toxin is botulinum toxin type B, preferably, the quantity of the botulinum toxin associated with the carrier is between about 100 units and about 30,000 units of a botulinum toxin type B.

Brief Summary Text (88):

A detailed embodiment of the present invention can comprise a controlled release system, comprising a biodegradable polymer and between about 10 units and about 100,000 units of a botulinum toxin encapsulated by the polymer carrier, thereby forming a controlled release system, wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a pulsatile manner in vivo upon subdermal implantation of the controlled release system in a human patient over a prolonged period of time extending from about 2 months to about 5 years.

Brief Summary Text (89):

A method for making an implant within the scope of the present invention can have the steps of: dissolving a polymer in a solvent to form a polymer solution; mixing or dispersing a botulinum toxin in the polymer solution to form a polymer-botulinum toxin mixture, and; allowing the polymer-botulinum toxin mixture to set or cure, thereby making an implant for pulsatile release of the botulinum toxin. This method can have the further step after the mixing step of evaporating solvent.

Brief Summary Text (90):

A method for using a pulsatile implant within the scope of the present invention can be by injecting or implanting a polymeric implant which includes a botulinum toxin, thereby treating a movement disorder or a disorder influenced by cholinergic innervation by local administration of a botulinum toxin.

Brief Summary Text (91):

An alternate embodiment of the present invention can be a carrier comprising a polymer selected from the group of polymers consisting of polylactides and polyglycolides and a stabilized botulinum toxin associated with the carrier, thereby thereby forming a pulsatile release botulinum toxin delivery system, wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a plurality of pulses in vivo upon subdermal implantation of the delivery system in a human patient. The carrier can comprise a plurality of discrete sets of polymeric, botulinum toxin incorporating microspheres, wherein each set of polymers has a different polymeric composition.

Brief Summary Text (92):

The botulinum toxin used in an implant according to the present invention can comprise: a first element comprising a binding element able to specifically bind to a neuronal cell surface receptor under physiological conditions, a second element comprising a translocation element able to facilitate the transfer of a polypeptide across a neuronal cell membrane, and a third element comprising a therapeutic element able, when present in the cytoplasm of a neuron, to inhibit exocytosis of acetylcholine from the neuron. The therapeutic element can cleave a SNARE protein, thereby inhibiting the exocytosis of acetylcholine from the neuron and the SNARE protein is can be selected from the group consisting of syntaxin, SNAP-25 and VAMP. Generally, the neuron affected by the botulinum toxin is a presynaptic, cholinergic, cholinergic, peripheral motor neuron.

Brief Summary Text (93):

The amount of a botulinum toxin administered by a continuous release system within the scope of the present invention during a given period can be between about 10.sup.-3 U/kg and about 35 U/kg for a botulinum toxin type A and up to about 2000

U/kg for other botulinum toxins, such as a botulinum toxin type B. 35 U/kg or 2000 U/kg is an upper limit because it approaches a lethal dose of certain neurotoxins, such as botulinum toxin type A or botulinum toxin type B, respectively. Thus, it has been reported that about 2000 units/kg of a commercially available botulinum toxin type B preparation approaches a primate lethal dose of type B botulinum toxin. Meyer K. E. et al, A Comparative Systemic Toxicity Study of Neurobloc in Adult Juvenile Cynomolgus Monkeys, Mov. Disord 15(Suppl 2);54;2000.

Brief Summary Text (94):

Preferably, the amount of a type A botulinum toxin administered by a continuous release system during a given period is between about 10.sup.-2 U/kg and about 25 U/kg. Preferably, the amount of a type B botulinum toxin administered by a continuous release system during a given period is between about 10.sup.-2 U/kg and about 1000 U/kg, since it has been reported that less than about 1000 U/kg of type B botulinum toxin can be intramuscularly administered to a primate without systemic effect. Ibid. More preferably, the type A botulinum toxin is administered in an amount of between about 10.sup.-1 U/kg and about 15 U/kg. Most preferably, the type A botulinum toxin is administered in an amount of between about 1 U/kg and about 10 U/kg. In many instances, an administration of from about 1 units to about 500 units of a botulinum toxin type A, provides effective and long lasting therapeutic relief. More preferably, from about 5 units to about 300 units of a botulinum toxin, such as a botulinum toxin type A, can be used and most preferably, from about about 10 units to about 200 units of a neurotoxin, such as a botulinum toxin type A, A, can be locally administered into a target tissue with efficacious results. In a particularly preferred embodiment of the present invention from about 1 units to about 100 units of a botulinum toxin, such as botulinum toxin type A, can be locally administered into a target tissue with therapeutically effective results.

Brief Summary Text (95):

The botulinum toxin can be made by Clostridium botulinum. Additionally, the botulinum toxin can be a modified botulinum toxin, that is a botulinum toxin that has at least one of its amino acids deleted, modified or replaced, as compared to the native or wild type botulinum toxin. Furthermore, the botulinum toxin can be a recombinant produced botulinum toxin or a derivative or fragment thereof.

Brief Summary Text (96):

Significantly, the botulinum toxin can be is administered to by subdermal implantation to the patient by placement of a botulinum toxin implant. The botulinum toxin can administered to a muscle of a patient in an amount of between about 1 unit and about 10,000 units. When the botulinum toxin is botulinum toxin type A and the botulinum toxin can be administered to a muscle of the patient in an amount of between about 1 unit and about 100 units.

Brief Summary Text (97):

Notably, it has been reported that glandular tissue treated by a botulinum toxin can show a reduced secretory activity for as long as 27 months post injection of the toxin. Laryngoscope 1999; 109:1344-1346, Laryngoscope 1998;108:381-384.

Brief Summary Text (98):

The present invention relates to an implant for the controlled release of a neurotoxin and to methods for making and using such implants. The implant can comprise a polymer matrix containing a botulinum toxin. The implant is designed to administer effective levels of neurotoxin over a prolonged period of time when administered, for example, intramuscularly, epidurally or subcutaneously for the treatment of various diseases conditions.

Brief Summary Text (99):

This invention further relates to a composition, and methods of making and using the composition, for the controlled of biologically active, stabilized neurotoxin. The controlled release composition of this invention can comprise a polymeric

matrix of a biocompatible polymer and biologically active, stabilized neurotoxin dispersed within the biocompatible polymer.

Brief Summary Text (104):

"Biologically active compound" means a compound which can effect a beneficial change in the subject to which it is administered. For example, "biologically active compounds" include neurotoxins.

Brief Summary Text (110):

"Neurotoxin" means an agent which can interrupt nerve impulse transmission across a neuromuscular or neuroglandular junction, block or reduce neuronal exocytosis of a neurotransmitter or alter the action potential at a sodium channel voltage gate of a neuron. Examples of neurotoxins include botulinum toxins, tetanus toxins, saxitoxins, and tetrodotoxin.

Brief Summary Text (112):

A method for making an implant within the scope of the present invention for controlled release of a neurotoxin, can include dissolving a biocompatible polymer in a polymer solvent to form a polymer solution, dispersing particles of biologically active, stabilized neurotoxin in the polymer solution, and then solidifying the polymer to form a polymeric matrix containing a dispersion of the neurotoxin particles.

Brief Summary Text (113):

A method of using an implant within the scope of the present invention forming for controlled release of a neurotoxin can comprise providing a therapeutically effective level of biologically active, neurotoxin in a patient for a prolonged period of time by implanting in the patient the implant.

Brief Summary Text (115):

The present invention is based upon the discovery of a pulsatile release implant comprising a biocompatible, biodegradable polymer capable of exhibiting in vivo multiphasic release of therapeutic amounts of a botulinum toxin over a prolonged period of time.

Brief Summary Text (116):

A botulinum toxin delivery system within the scope of the present invention is capable of pulsatile (i.e. multiphasic) release of therapeutic amounts of a botulinum toxin. By pulsatile release it is meant that during a period of time, which can extend from about 1 hour to about 4 weeks, a quantity of therapeutically effective (i.e. biologically active) botulinum toxin is released from a carrier material in vivo at the site of implantation. The pulse of released botulinum toxin can comprise (for a botulinum toxin type A) as little as about 1 unit (i.e. to treat treat blepharospasm) to as much as 200 units (i.e. to treat of a large spasmodic muscle, such as the biceps). The quantity of botulinum toxin required for therapeutic efficacy can be varied according to the known clinical potency of the different botulinum toxin serotypes. For example, several orders of magnitude more units of a botulinum toxin type B are typically required to achieve a physiological effect comparable to that achieved from use of a botulinum toxin type A. Prior to and following each pulse there is a period of reduced or substantially no botulinum toxin release from the implant.

Brief Summary Text (117):

The botulinum toxin released in therapeutically effective amounts by a controlled release delivery system within the scope of the present invention is preferably, substantially biologically active botulinum toxin. In other words, the botulinum toxin released from the disclosed delivery system is capable of binding with high affinity to a cholinergic neuron, being translocated, at least in part, across the neuronal membrane, and through its activity in the cytosol of the neuron of inhibiting exocytosis of acetylcholine from the neuron. The present invention

excludes from its scope use deliberate use of a botulinum toxoid as an antigen in order to confer immunity to the botulinum toxin through development of antibodies (immune response) due to the immunogenicity of the toxoid. The purpose of the present invention is to permit a controlled release of minute amounts of a botulinum toxin from a delivery system so as to inhibit exocytosis in vivo and thereby achieve a desired therapeutic effect, such as reduction of muscle spasm or muscle tone, preventing a muscle from contracting or to reduce an excessive secretion (i.e. a sweat secretion) from a cholinergically influenced secretory cell or gland.

Brief Summary Text (118):

Pulsatile release of a botulinum toxin from an implant can be accomplished by preparing a plurality of implants with differing carrier material compositions. For example, holding other factors, such as polymer molecular weight, constant an implant can be made up of a several sets of botulinum toxin encapsulated microspheres, each set of microspheres having a different polymer composition such that the polymers of each set of microspheres degrade, and release toxin, at differing rates. Conveniently, the plurality of sets of differing polymer composition microspheres can be pressed into the form of a disc, and implanted as a pellet. The pulsatile release implant can be implanted subcutaneously, intramuscularly, intracranially, intraglandular, etc, at a site so that systemic entry of the toxin is not encouraged.

Brief Summary Text (119):

A first pulse of a botulinum toxin can be locally administered due to the presence of a botulinum toxin (i.e. free or non-implant incorporated botulinum toxin) administered in conjunction with and at the same time as insertion of the implant and/or due to a burst effect of botulinum toxin release from the implanted microspheres. A second pulse of a botulinum toxin can be administered by the implant at about three months post implantation upon biodegradation of a first set of microspheres. A third pulse of a botulinum toxin can be delivered by the system at about six months post implantation upon dissolution of a second set of bioerodible microspheres, and so on. Thus, a botulinum toxin delivery system within the scope of the present invention which comprises three differing sets of appropriate microsphere polymer compositions, permits a patient to be reimplant or reinvested with a botulinum toxin only once every 12 months.

Brief Summary Text (120):

For example, it is known that biodegradable PLA:PGA microspheres can be made with varying copolymer content such that proportionally different polymer degradation time windows result. Thus, a 75:25 lactide:glycolide polymer can degrade at about ninety days post implantation. Additionally, a 100:0 lactide:glycolide polymer can degrade at about one hundred and eighty days post implantation. Furthermore, a 95:5 poly(DL-lactide):glycolide polymer can degrade at about two hindered and seventy days post implantation. Finally, a 100:0 poly(DL-lactide):glycolide polymer can degrade at about twelve months post implantation. See e.g. Kissel et al, Microencapsulation of Antigens Using Biodegradable Polymers: Facts and Fantasies, Behring Inst. Mitt., 98;172-183:1997; Cleland J. L., et al, Development of a Single-Shot Subunit Vaccine for HOV-1: Part 4. Optimizing Microencapsulation and Pulsatile Release of MN rpg120 from Biodegradable Microspheres, J Cont Rel 47;135-150:1997, and; Lewis D. H., Controlled Release of Bioactive Agents from Lactide/Glycolide Polymers, pages 1-41 of Chasin M., et al, "Biodegradable Polymers as Drug-Delivery Systems", Marcel Dekker, New York (1990). The above-specified four discrete sets of polymeric microspheres can be prepared as botulinum toxin incorporating microspheres, and combined into a single implant capable of pulsatile release of the botulinum toxin over a one year period, thereby providing a patient treatment period per implant of about 15-16 months.

Brief Summary Text (121):

The delivery system is prepared so that the botulinum toxin is substantially

uniformly dispersed in a biodegradable carrier. An alternate pulsatile delivery system within the scope of the present invention can comprise a carrier coated by a biodegradable coating, either the thickness of the coating or the coating material being varied, such that in the different sets of microspheres, the respective coating take from 3, 6, 9, etc months to be dissolved, thereby providing the desired desired toxin pulses. The microspheres are inert and are of such a size or due to being pressed into a disc, that they do not diffuse significantly beyond the site of injection. Hence, multiple implantations, as by needle injection, can be carried out out at the same time.

Brief Summary Text (122):

A third embodiment within the scope of the present invention of a pulsatile, implant can comprise a non-porous, non-biodegradable, biocompatible tube which is closed at one end. Carrier associated neurotoxin is interspaced discrete locations within the bore of the tube. Thus, toxin at an open or porous, or erodible plug sealed pug the end of the tube rapidly diffuses out, causing the first local administration. Toxin further from the end of the tube takes longer to diffuse out and results in the second local.

Brief Summary Text (123):

The thickness of the implant can be used to control the absorption of water by, and thus the rate of release of a neurotoxin from, a composition of the invention, thicker implants releasing the polypeptide more slowly than thinner ones.

Brief Summary Text (124):

The neurotoxin in a neurotoxin controlled release composition can also be mixed with with other excipients, such as bulking agents or additional stabilizing agents, such as buffers to stabilize the neurotoxin during lyophilization.

Brief Summary Text (127):

Biodegradable PLGA polymers have been used to form resorbable sutures and bone plates and in several commercial microparticle formulations. PLGA degrades through bulk erosion to produce lactic and glycolic acid and is commercially available in a variety of molecular weight and polymer end groups (e.g. lauryl alcohol or free acid). Polyanhydrides are another group of polymers that have been approved for use I humans, and have been used to deliver proteins and antigens. Unlike PLGA, polyanhydrides degrade by surface erosion, releasing neurotoxin entrapped at the carrier surface.

Brief Summary Text (128):

To prepare a suitable implant, the carrier polymer is dissolved in an organic solvent such as methylene chloride or ethyl acetate and the botulinum toxin is then mixed into the polymer solution. The conventional processes for microsphere formation are solvent evaporation and solvent (coacervation) methods. The water-in-oil-in-water (W/O/W) double emulsion method is a widely used method of protein antigen encapsulation into PLGA microspheres.

Brief Summary Text (129):

An aqueous solution of a botulinum toxin can be used to make a pulsatile implant. An An aqueous solution of the neurotoxin is added to the polymer solution (polymer previously dissolved in a suitable organic solvent). The volume of the aqueous (neurotoxin) solution relative to the volume of organic (polymer) solvent is an important parameter in the determination of both the release characteristics of the microspheres and with regard to the encapsulation efficiency (ratio of theoretical to experimental protein loading) of the neurotoxin.

Brief Summary Text (131):

Thus, with a low aqueous phase (neurotoxin) to organic phase (polymer) volume ratio (i.e. aqueous volume:organic volume is .1toeq.0.1 ml/ml) essentially 100% of the neurotoxin can be encapsulated by the microspheres and the microspheres can show a

triphasic release: an initial burst (first pulse), a lag phase with little or no neurotoxin being released and a second release phase (second pulse).

Brief Summary Text (132):

The length of the lag phase is dependent upon the polymer degradation rate which is in turn dependant upon polymer composition and molecular weight. Thus, the lag phase between the first (burst) pulse and the second pulse increases as the lactide content is increased, or as the polymer molecular weight is increased with the lactide:glycolide ratio being held constant. In addition to a low aqueous phase (neurotoxin) volume, operation at low temperature (2-8 degrees C.), as set forth above, increases the encapsulation efficiency, as well as reducing the initial burst and promoting increased neurotoxin stability against thermal inactivation.

Brief Summary Text (133):

Suitable implants within the scope of the present invention for the controlled in vivo release of a neurotoxin, such as a botulinum toxin, can be prepared so that the the implant releases the neurotoxin in a pulsatile manner. A pulsatile release implant can release a neurotoxin in a biphasic or multiphase manner. Thus, a pulsatile release implant can have a relatively short initial induction (burst) period, followed by periods during which reduced, little or no neurotoxin is released.

Brief Summary Text (134):

A controlled release of biologically active neurotoxin is a release which results in therapeutically effective, with negligible serum levels, of biologically active, neurotoxin over a period longer than that obtained following direct administration of aqueous neurotoxin. It is preferred that a controlled release be a release of neurotoxin for a period of about six months or more, and more preferably for a period of about one year or more.

Brief Summary Text (136):

Denaturation of the encapsulated neurotoxin in the body at 37 degrees C. for a prolonged period of time can be reduced by stabilizing the neurotoxin by lyophilizing it with albumin, lyophilizing from an acidic solution, lyophilizing from a low moisture content solution (these three criteria can be met with regard to a botulinum toxin type A by use of non-reconstituted Botox.RTM.) and using a specific polymer matrix composition.

Brief Summary Text (137):

Preferably, the release of biologically active neurotoxin in vivo does not result in a significant immune system response during the release period of the neurotoxin.

Brief Summary Text (138):

A pulsatile botulinum toxin delivery system preferably permits botulinum release from biodegradable polymer microspheres in a biologically active form, that is with a substantially native toxin conformation. To stabilize a neurotoxin, both in a format which renders the neurotoxin useful for mixing with a suitable polymer which can form the implant matrix (i.e. a powdered neurotoxin which has been freeze dried or lyophilized) as well as while the neurotoxin is present or incorporated into the matrix of the selected polymer, various pharmaceutical excipients can be used. Suitable excipients can include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, albumin and dried skim milk. The neurotoxin in a neurotoxin controlled release composition can be mixed with excipients, bulking agents and stabilizing agents, and buffers to stabilize the neurotoxin during lyophilization or freeze drying.

Brief Summary Text (139):

It has been discovered that a stabilized neurotoxin can comprise biologically

active, non-aggregated neurotoxin complexed with at least one type of multivalent metal cation which has a valiancy of +2 or more.

Brief Summary Text (141):

Preferably, the molar ratio of metal cation component to neurotoxin, for the metal cation stabilizing the neurotoxin, is between about 4:1 to about 100:1 and more typically about 4:1 to about 10:1.

Brief Summary Text (142):

A preferred metal cation used to stabilize a botulinum toxin is Zn.sup.++ because the botulinum toxin are known to be zinc endopeptidases. Divalent zinc cations are preferred because botulinum toxin is known to be a divalent zinc endopeptidase. In a more preferred embodiment, the molar ratio of metal cation component, containing Zn.sup.++ cations, to neurotoxin is about 6:1.

Brief Summary Text (143):

The suitability of a metal cation for stabilizing neurotoxin can be determined by one of ordinary skill in the art by performing a variety of stability indicating techniques such as polyacrylamide gel electrophoresis, isoelectric focusing, reverse phase chromatography, HPLC and potency tests on neurotoxin lyophilized particles containing metal cations to determine the potency of the neurotoxin after lyophilization and for the duration of release from microparticles. In stabilized neurotoxin, the tendency of neurotoxin to aggregate within a microparticle during hydration in vivo and/or to lose biological activity or potency due to hydration or due to the process of forming a controlled release composition, or due to the chemical characteristics of a controlled release composition, is reduced by complexing at least one type of metal cation with neurotoxin prior to contacting the neurotoxin with a polymer solution.

Brief Summary Text (144):

By the present invention, stabilized neurotoxin is stabilized against significant aggregation in vivo over the controlled release period. Significant aggregation is defined as an amount of aggregation resulting in aggregation of about 15% or more of the polymer encapsulated or polymer matrix incorporated neurotoxin. Preferably, aggregation is maintained below about 5% of the neurotoxin. More preferably, aggregation is maintained below about 2% of the neurotoxin present in the polymer.

Brief Summary Text (145):

In another embodiment, a neurotoxin controlled release composition also contains a second metal cation component, which is not contained in the stabilized neurotoxin particles, and which is dispersed within the polymer. The second metal cation component preferably contains the same species of metal cation, as is contained in the stabilized neurotoxin. Alternately, the second metal cation component can contain one or more different species of metal cation.

Brief Summary Text (146):

The second metal cation component acts to modulate the release of the neurotoxin from the polymeric matrix of the controlled release composition, such as by acting as a reservoir of metal cations to further lengthen the period of time over which the neurotoxin is stabilized by a metal cation to enhance the stability of neurotoxin in the composition.

Brief Summary Text (147):

A metal cation component used in modulating release typically contains at least one type of multivalent metal cation. Examples of second metal cation components suitable to modulate neurotoxin release, include, or contain, for instance, Mg(OH).sub.2, MgCO.sub.3 (such as 4MgCO.sub.3 Mg(OH).sub.2 5H.sub.2 O), ZnCO.sub.3 (such as 3Zn(OH).sub.2 2ZnCO.sub.3), CaCO.sub.3, Zn.sub.3 (C.sub.6 H.sub.5 O.sub.7).sub.2, Mg(OAc).sub.2, MgSO.sub.4, Zn(OAc).sub.2, ZnSO.sub.4, ZnCl.sub.2, MgCl.sub.2 and Mg.sub.3 (C.sub.6 H.sub.5 O.sub.7).sub.2. A suitable ratio of second

metal cation component-to-polymer is between about 1:99 to about 1:2 by weight. The optimum ratio depends upon the polymer and the second metal cation component utilized.

Brief Summary Text (148):

The neurotoxin controlled release composition of this invention can be formed into many shapes such as a film, a pellet, a cylinder, a disc or a microsphere. A microsphere, as defined herein, comprises a polymeric component having a diameter of less than about one millimeter and having stabilized neurotoxin dispersed therein. A microsphere can have a spherical, non-spherical or irregular shape. It is preferred that a microsphere be spherical in shape. Typically, the microsphere will be of a size suitable for injection. A preferred size range for microspheres is from about 1 to about 180 microns in diameter.

Brief Summary Text (149):

In the method of this invention for forming a composition for the controlled release of biologically active, non-aggregated neurotoxin, a suitable amount of particles of biologically active, stabilized neurotoxin are dispersed in a polymer solution.

Brief Summary Text (150):

A suitable polymer solvent, as defined herein, is solvent in which the polymer is soluble but in which the stabilized neurotoxin is are substantially insoluble and non-reactive. Examples of suitable polymer solvents include polar organic liquids, such as methylene chloride, chloroform, ethyl acetate and acetone.

Brief Summary Text (151):

To prepare biologically active, stabilized neurotoxin, neurotoxin is mixed in a suitable aqueous solvent with at least one suitable metal cation component under pH conditions suitable for forming a complex of metal cation and neurotoxin. Typically, the complexed neurotoxin will be in the form of a cloudy precipitate, which is suspended in the solvent. However, the complexed neurotoxin can also be in solution. In an even more preferred embodiment, neurotoxin is complexed with Zn.sup.++.

Brief Summary Text (152):

Suitable pH conditions to form a complex of neurotoxin typically include pH values between about 5.0 and about 6.9. Suitable pH conditions are typically achieved through use of an aqueous buffer, such as sodium bicarbonate, as the solvent.

Brief Summary Text (153):

Suitable solvents are those in which the neurotoxin and the metal cation component are each at least slightly soluble, such as in an aqueous sodium bicarbonate buffer. For aqueous solvents, it is preferred that water used be either deionized water or water-for-injection (WFI).

Brief Summary Text (154):

The neurotoxin can be in a solid or a dissolved state, prior to being contacted with with the metal cation component. Additionally, the metal cation component can be in a solid or a dissolved state, prior to being contacted with the neurotoxin. In a preferred embodiment, a buffered aqueous solution of neurotoxin is mixed with an aqueous solution of the metal cation component.

Brief Summary Text (155):

Typically, the complexed neurotoxin will be in the form of a cloudy precipitate, which is suspended in the solvent. However, the complexed neurotoxin can also be in solution. In a preferred embodiment, the neurotoxin is complexed with Zn.sup.++.

Brief Summary Text (156):

The Zn.sup.++ complexed neurotoxin can then be dried, such as by lyophilization, to

form particulates of stabilized neurotoxin. The Zn.sup.++ complexed neurotoxin, which is suspended or in solution, can be bulk lyophilized or can be divided into smaller volumes which are then lyophilized. In a preferred embodiment, the Zn.sup.++ complexed neurotoxin suspension is micronized, such as by use of an ultrasonic nozzle, and then lyophilized to form stabilized neurotoxin particles. Acceptable means to lyophilize the Zn.sup.++ complexed neurotoxin mixture include those known in the art.

Brief Summary Text (157):

In another embodiment, a second metal cation component, which is not contained in the stabilized neurotoxin particles, is also dispersed within the polymer solution.

Brief Summary Text (158):

It is understood that a second metal cation component and stabilized neurotoxin can be dispersed into a polymer solution sequentially, in reverse order, intermittently, separately or through concurrent additions. Alternately, a polymer, a second metal cation component and stabilized neurotoxin and can be mixed into a polymer solvent sequentially, in reverse order, intermittently, separately or through concurrent additions. In this method, the polymer solvent is then solidified to form a polymeric matrix containing a dispersion of stabilized neurotoxins.

Brief Summary Text (159):

A suitable method for forming an neurotoxin controlled release composition from a polymer solution is the solvent evaporation method is described in U.S. Pat. Nos. 3,737,337; 3,523,906; 3,691,090, and; 4,389,330. Solvent evaporation can be used as a method to form neurotoxin controlled release microparticles.

Brief Summary Text (160):

In the solvent evaporation method, a polymer solution containing a stabilized neurotoxin particle dispersion, is mixed in or agitated with a continuous phase, in which the polymer solvent is partially miscible, to form an emulsion. The continuous phase is usually an aqueous solvent. Emulsifiers are often included in the continuous phase to stabilize the emulsion. The polymer solvent is then evaporated over a period of several hours or more, thereby solidifying the polymer to form a polymeric matrix having a dispersion of stabilized neurotoxin particles contained therein.

Brief Summary Text (161):

A preferred method for forming neurotoxin controlled release microspheres from a polymer solution is described in U.S. Pat. No. 5,019,400. This method of microsphere formation, as compared to other methods, such as phase separation, additionally reduces the amount of neurotoxin required to produce a controlled release composition with a specific neurotoxin content.

Brief Summary Text (162):

In this method, the polymer solution, containing the stabilized neurotoxin dispersion, is processed to create droplets, wherein at least a significant portion of the droplets contain polymer solution and the stabilized neurotoxin. These droplets are then frozen by means suitable to form microspheres. Examples of means for processing the polymer solution dispersion to form droplets include directing the dispersion through an ultrasonic nozzle, pressure nozzle, Rayleigh jet, or by other known means for creating droplets from a solution.

Brief Summary Text (163):

The solvent in the frozen microdroplets is extracted as a solid and/or liquid into the non-solvent to form stabilized neurotoxin containing microspheres. Mixing ethanol with other non-solvents, such as hexane or pentane, can increase the rate of solvent extraction, above that achieved by ethanol alone, from certain polymers,

such as poly(lactide-co-glycolide) polymers.

Brief Summary Text (164):

Yet another method of forming a neurotoxin implant, from a polymer solution, includes film casting, such as in a mold, to form a film or a shape. For instance, after putting the polymer solution containing a dispersion of stabilized neurotoxin into a mold, the polymer solvent is then removed by means known in the art, or the temperature of the polymer solution is reduced, until a film or shape, with a consistent dry weight, is obtained.

Brief Summary Text (165):

In the case of a biodegradable polymer implant, release of neurotoxin due to degradation of the polymer. The rate of degradation can be controlled by changing polymer properties that influence the rate of hydration of the polymer. These properties include, for instance, the ratio of different monomers, such as lactide and glycolide, comprising a polymer; the use of the L-isomer of a monomer instead of a racemic mixture; and the molecular weight of the polymer. These properties can affect hydrophilicity and crystallinity, which control the rate of hydration of the polymer. Hydrophilic excipients such as salts, carbohydrates and surfactants can also be incorporated to increase hydration and which can alter the rate of erosion of the polymer.

Brief Summary Text (166):

By altering the properties of a biodegradable polymer, the contributions of diffusion and/or polymer degradation to neurotoxin release can be controlled. For example, increasing the glycolide content of a poly(lactide-co-glycolide) polymer and decreasing the molecular weight of the polymer can enhance the hydrolysis of the polymer and thus, provides an increased neurotoxin release from polymer erosion. In addition, the rate of polymer hydrolysis is increased in non-neutral pH's. Therefore, an acidic or a basic excipient can be added to the polymer solution, used to form the microsphere, to alter the polymer erosion rate.

Brief Summary Text (167):

An implant within the scope of the present invention can be administered to a human, or other animal, by any non-systemic means of administration, such as by implantation (e.g. subcutaneously, intramuscularly, intracranially, intravaginally and intradermally), to provide the desired dosage of neurotoxin based on the known parameters for treatment with neurotoxin of various medical conditions, as previously set forth.

Brief Summary Text (168):

The specific dosage by implant appropriate for administration is readily determined by one of ordinary skill in the art according to the factor discussed above. The dosage can also depend upon the size of the tissue mass to be treated or denervated, and the commercial preparation of the toxin. Additionally, the estimates for appropriate dosages in humans can be extrapolated from determinations of the amounts of botulinum required for effective denervation of other tissues. Thus, the amount of botulinum A to be injected is proportional to the mass and level of activity of the tissue to be treated. Generally, between about 0.01 units per kilogram to about 35 units per kg of patient weight of a botulinum toxin, such as botulinum toxin type A, can be released by the present implant per unit time period (i.e. over a period of or once every 2-4 months) to effectively accomplish a desired muscle paralysis. Less than about 0.01 U/kg of a botulinum toxin does not have a significant therapeutic effect upon a muscle, while more than about 35 U/kg of a botulinum toxin approaches a toxic dose of a neurotoxin, such as a botulinum toxin type A. Careful preparation and placement of the implant prevents significant amounts of a botulinum toxin from appearing systemically. A more preferred dose range is from about 0.01 U/kg to about 25 U/kg of a botulinum toxin, such as that formulated as BOTOX.RTM.. The actual amount of U/kg of a botulinum toxin to be administered depends upon factors such as the extent (mass) and level of activity

of the tissue to be treated and the administration route chosen. Botulinum toxin type A is a preferred botulinum toxin serotype for use in the methods of the present present invention.

Brief Summary Text (169):

Preferably, a neurotoxin used to practice a method within the scope of the present invention is a botulinum toxin, such as one of the serotype A, B, C, D, E, F or G botulinum toxins. Preferably, the botulinum toxin used is botulinum toxin type A, because of its high potency in humans, ready availability, and known safe and efficacious use for the treatment of skeletal muscle and smooth muscle disorders when locally administered by intramuscular injection.

Brief Summary Text (170):

The present invention includes within its scope the use of any neurotoxin which has a long duration therapeutic effect when used to treat a movement disorder or an affliction influenced by cholinergic innervation. For example, neurotoxins made by any of the species of the toxin producing Clostridium bacteria, such as Clostridium botulinum, Clostridium butyricum, and Clostridium beratti can be used or adapted for for use in the methods of the present invention. Additionally, all of the botulinum serotypes A, B, C, D, E, F and G can be advantageously used in the practice of the present invention, although type A is the most preferred serotype, as explained above. Practice of the present invention can provide effective relief for from 1 month to about 5 or 6 years.

Brief Summary Text (171):

The present invention includes within its scope: (a) neurotoxin complex as well as pure neurotoxin obtained or processed by bacterial culturing, toxin extraction, concentration, preservation, freeze drying and/or reconstitution and; (b) modified or recombinant neurotoxin, that is neurotoxin that has had one or more amino acids or amino acid sequences deliberately deleted, modified or replaced by known chemical/biochemical amino acid modification procedures or by use of known host cell/recombinant vector recombinant technologies, as well as derivatives or fragments of neurotoxins so made, and includes neurotoxins with one or more attached attached targeting moieties for a cell surface receptor present on a cell.

Brief Summary Text (172):

Botulinum toxins for use according to the present invention can be stored in lyophilized or vacuum dried form in containers under vacuum pressure. Prior to lyophilization the botulinum toxin can be combined with pharmaceutically acceptable excipients, stabilizers and/or carriers, such as albumin. The lyophilized or vacuum dried material can be reconstituted with saline or water.

Brief Summary Text (173):

The present invention also includes within its scope the use of an implanted controlled release neurotoxin complex so as to provide therapeutic relief from a chronic disorder such as movement disorder. Thus, the neurotoxin can be imbedded within, absorbed, or carried by a suitable polymer matrix which can be implanted or embedded subdermally so as to provide a year or more of delayed and controlled release of the neurotoxin to the desired target tissue. Implantable polymers which permit controlled release of polypeptide drugs are known, and can be used to prepare a botulinum toxin implant suitable for insertion or subdermal attachment. See e.g. Pain 1999;82(1):49-55; Biomaterials 1994;15(5):383-9; Brain Res 1990;515(1-2):309-11 and U.S. Pat. Nos. 6,022,554; 6,011,011; 6,007,843; 5,667,808, and; 5,980,945.

Brief Summary Text (174):

Methods for determining the appropriate route of administration and dosage are generally determined on a case by case basis by the attending physician. Such determinations are routine to one of ordinary skill in the art (see for example, Harrison's Principles of Internal Medicine (1998), edited by Anthony Fauci et al.,

14.sup.th edition, published by McGraw Hill). Thus, an implant within the scope of the present invention can be surgically inserted by incision at the site of desired effect (i.e. for reduction of a muscle spasm) or the implant can be administered as a suspension, subcutaneously or intramuscularly using a hollow needle implanting gun, for example of the type disclosed in U.S. Pat. No. 4,474,572. The diameter of the needle may be adjusted to correspond to the size of the implant used. Further, an implant within the scope of the present invention can be implanted intracranially intracranially so as to provide long term delivery of a therapeutic amount of a neurotoxin to a target brain tissue. Removal of a non-biodegradable implant within the scope of the present invention is not essential once all neurotoxin has been released due to the biocompatible, nonimmunogenic nature of the implant materials used.

Brief Summary Text (175):

It is known that a significant water content of lyophilized tetanus toxoid can cause solid phase aggregation and inactivation of the toxoid once encapsulated within microspheres. Thus, with a 10% (grams of water per 100 grams of protein) tetanus toxoid water content about 25% of the toxin undergoes aggregation, while with a 5% water content only about 5% of the toxoid aggregates. See e.g. Pages 251, Schwendeman S. P. et al., Peptide, Protein, and Vaccine Delivery From Implantable Polymeric Systems, chapter 12 (pages 229-267) of Park K., Controlled Drug Delivery Challenges and Strategies, American Chemical Society (1997). Significantly, the manufacturing process for BOTOX.RTM. results in a freeze dried botulinum toxin type A complex which has a moisture content of less than about 3%, at which moisture level nominal solid phase aggregation can be expected.

Brief Summary Text (176):

A general procedure for making a pulsatile, biodegradable botulinum toxin implant is as follows. The implant can comprise from about 25% to about 100% of a polylactide which is a polymer of lactic acid alone. Increasing the amount of lactide in the implant can increase the period of time before which the implant begins to biodegrade, and hence increase the time to pulsatile release of the botulinum toxin from the implant. The implant can also be a copolymer of lactic acid and glycolic acid. The lactic acid can be either in racemic or in optically active form, and can be either soluble in benzene and having an inherent viscosity of from 0.093 (1 g. per 100 ml. in chloroform) to 0.5 (1 g. per 100 ml. in benzene), or insoluble in benzene and having an inherent viscosity of from 0.093 (1 g. per 100 ml in chloroform) to 4 (1 g. per 100 ml in chloroform or dioxin). The implant can also comprise from 0.001% to 50% of a botulinum toxin uniformly dispersed in carrier polymer.

Brief Summary Text (177):

Once implanted the implant begins to absorb water and exhibits two successive and generally distinct phases of neurotoxin release. In the first phase neurotoxin is released through by initial diffusion through aqueous neurotoxin regions which communicate with the exterior surface of the implant. The second phase occurs upon release of neurotoxin consequent to degradation of the biodegradable polymer (i.e. a polylactide). The diffusion phase and the degradation-induced phase are temporally distinct in time. When the implant is placed in an aqueous physiological environment, water diffuses into the polymeric matrix and is partitioned between neurotoxin and polylactide to form aqueous neurotoxin regions. The aqueous neurotoxin regions increase with increasing absorption of water, until the continuity of the aqueous neurotoxin regions reaches a sufficient level to communicate with the exterior surface of the implant. Thus, neurotoxin starts to be released from the implant by diffusion through aqueous polypeptide channels formed from the aqueous neurotoxin regions, while the second phase continues until substantially all of the remaining neurotoxin has been released.

Brief Summary Text (178):

Also within the scope of the present invention is an implant in the form of a

suspension for use by injection, prepared by suspending the neurotoxin encapsulated microspheres in a suitable liquid, such as physiological saline.

Detailed Description Text (4):

Method for Making a Biodegradable Botulinum Toxin Implant

Detailed Description Text (5):

A biodegradable implant comprising botulinum toxin and a suitable carrier polymer can be prepared by dispersing an appropriate amount of a stabilized botulinum toxin preparation (i.e. non-reconstituted BOTOX.RTM.) into a continuous phase consisting of a biodegradable polymer in a volatile organic solvent, such as dichloromethane. Both PLGA and polyanhydrides are insoluble in water and require use of organic solvents in the microencapsulation process.

Detailed Description Text (6):

The polymer is dissolved in an organic solvent such as methylene chloride or ethyl acetate to facilitate microsphere fabrication. The botulinum toxin is then mixed by homogenization or sonication to form a fine dispersion of toxin in polymer/organic solvent, as an emulsion when an aqueous protein solution is used or as a suspension when a solid protein formulation is mixed with the polymer-organic solvent solution. The conventional processes for microsphere formation are solvent evaporation and solvent (coacervation) methods. Microspheres can be formed by mixing the preformed suspension of protein drug with polymer-organic solvent, with water containing an emulsifier (i.e. polyvinyl alcohol). Additional water is then added to facilitate removal of the organic solvent from the microspheres allowing them to harden. The final microspheres are dried to produce a free flowing powder.

Detailed Description Text (7):

The polymer used can be PLA, PGA or a co-polymer thereof. Alternately, a botulinum toxin incorporating polymer can be prepared by emulsifying an aqueous solution of the neurotoxin (i.e. reconstituted BOTOX.RTM.) into the polymerorganic phase (obtaining thereby a W/O emulsion). With either process a high speed stirrer or ultrasound is used to ensure uniform toxin mixing with the polymer. Microparticles 1-50 .mu.m in diameter can be formed by atomizing the emulsion into a stream of hot air, inducing the particle formation through evaporation of the solvent (spray-drying technique). Alternately, particle formation can be achieved by coacervation of the polymer through non-solvent addition, e.g. silicon oil (phase separation technique) or by preparing a W/O/W emulsion (double emulsion technique).

Detailed Description Text (8):

The pH of the casting or other solution in which the botulinum toxin is to be mixed is maintained at pH 4.2-6.8, because at pH above about pH 7 the stabilizing nontoxin proteins can dissociate from the botulinum toxin resulting in gradual loss of toxicity. Preferably, the pH is between about 5-6. Furthermore the temperature of the mixture/solution should not exceed about 35 degrees Celsius, because the toxin can be readily detoxified when in a solution/mixture heated above about 40 degrees Celsius.

Detailed Description Text (10):

A wide range of sizes of botulinum toxin implant microparticles can be made by varying the droplet size, for example, by changing the ultrasonic nozzle diameter. If very large microparticles are desired, the microparticles can be extruded through a syringe directly into the cold liquid. Increasing the viscosity of the polymer solution can also increase microparticle size. The size of the microparticles can be produced by this process, for example microparticles ranging from greater than about 1000 to about 1 micrometers in diameter.

Detailed Description Text (12):

Method for Making a Polyanhydrides Botulinum Toxin Implant

Detailed Description Text (13):

A biodegradable polyanhydride polymer can be made as a copolymer of polycarboxyphenoxypropane and sebacic acid in a ratio of 20:80. Polymer and a botulinum toxin (such as non-reconstituted BOTOX.RTM.) can be co-dissolved in methylene chloride at room temperature and spray-dried into microspheres, using the technique of Example 1. Any remaining methylene chloride can be evaporated in a vacuum desiccator.

Detailed Description Text (14):

Depending upon the implant size desired and hence the amount of botulinum toxin, a suitable amount of the microspheres can be compressed at about 8000 p.s.i. for 5 seconds or at 3000 p.s.i. for 17 seconds in a mold to form implant discs encapsulating the neurotoxin. Thus, the microspheres can be compression molded pressed into discs 1.4 cm in diameter and 1.0 mm thick, packaged in aluminum foil pouches under nitrogen atmosphere and sterilized by 2.2.times.10.sup.4 Gy gamma irradiation. The polymer permits release of the botulinum toxin over a prolonged period, and it can take more than a year for the polymer to be largely degraded.

Detailed Description Text (16):

Water in Oil Method for Making a Biodegradable Botulinum Toxin Implant

Detailed Description Text (17):

A pulsatile release botulinum toxin implant can be made by dissolving a 80:20 copolymers of polyglycolic acid and the polylactic acid can in 10% w/v of dichloromethane at room temperature with gentle agitation. A water-in-oil type emulsion can then be made by adding 88 parts of the polymer solution to 1 part of a 1:5 mixture of Tween 80 (polyoxyethylene 20 sorbitan monooleate, available from Acros Organics N.V., Fairlawn, N.J.) and Span 85 (sorbitan trioleate) and 11 parts of an aqueous mixture of 75 units of BOTOX.RTM. (botulinum toxin type A complex) and Quil A (adjuvant). The mixture is agitated using a high-speed blender and then immediately spray-dried using a Drytec Compact Laboratory Spray Dryer equipped with a 60/100/120 nozzle at an atomizing pressure of 15 psi and an inlet temperature of 65 degrees C. The resultant microspheres have a diameter of about 20 .mu.m diameter and are collected as a free-flowing powder. Traces of remaining organic solvent are removed by vacuum evaporation.

Detailed Description Text (19):

Reduced Temperature Method for a Biodegradable Pulsatile Botulinum Toxin Implant

Detailed Description Text (20):

A pulsatile release botulinum toxin delivery system can be made at a low temperature temperature so as to inhibit toxin denaturation as follows. 0.3 g of PLGA/ml of methylene chloride or ethyl acetate is mixed with 0.1 ml of neurotoxin solution/ml of the polymer-organic solution at a reduced temperature (2-8 degrees C.). A first set of botulinum toxin incorporating microspheres made, as set forth in Example 1 (the polymer solution is formed by dissolving the polymer in methylene chloride), from a 75:25 lactide:glycolide polymer with an inherent viscosity (dL/g) of about 0.62 (available form MTI) can degrade in vivo, and hence exhibit a pulsed release of the botulinum toxin, at about ninety days post implantation and extending over 2-2-4 weeks. A second set of, botulinum toxin incorporating microspheres made, as previously set forth (the polymer solution is formed by dissolving the polymer in ethyl acetate), from a 100:0 lactide:glycolide polymer with an inherent viscosity of about 0.22 (available form MTI) can degrade in vivo, and hence exhibit a burst release of the botulinum toxin, at about one hundred and eighty days post implantation. A third set of, botulinum toxin incorporating microspheres made, as previously set forth (the polymer solution is formed by dissolving the polymer in methylene chloride, from a 95:5 poly(DL-lactide):glycolide polymer, can degrade in vivo, and hence exhibit a burst release of the botulinum toxin, at about two hindered and seventy days post implantation. A fourth set of botulinum toxin incorporating microspheres made, as previously set forth (the polymer solution is

formed by dissolving the polymer in methylene chloride), from a 100:0 poly(DL-lactide):glycolide polymer can degrade in vivo, and hence exhibit a burst release of the botulinum toxin, at about twelve months post implantation. Polymers can be obtained from Medisorb Technologies International (MTI).

Detailed Description Text (21):

A suspension or compression molded pellet which combines the four specified sets of botulinum toxin encapsulated microspheres can exhibit pulsatile release the neurotoxin. Local administration of botulinum toxin at the time of implantation (i.e. day zero) is provided by the initial burst release from the implanted microspheres.

Detailed Description Text (23):

1. a single implant can be used to provide therapeutically effective continuous or pulsatile administration of a neurotoxin over a period of one year or longer.

Detailed Description Text (24):

2. the neurotoxin is delivered to a localized tissue area without a significant amount of neurotoxin appearing systemically.

Detailed Description Text (26):

4. reduced need for periodic injections of neurotoxin to treat a condition, such as a neuromuscular disorder.

Detailed Description Text (29):

An advantage of the present controlled release formulations for neurotoxins include long term, consistent therapeutic levels of neurotoxin at the target tissue. The advantages also include increased patient compliance and acceptance by reducing the required number of injections.

Detailed Description Text (31):

Although the present invention has been described in detail with regard to certain preferred methods, other embodiments, versions, and modifications within the scope of the present invention are possible. For example, a wide variety of neurotoxins can be effectively used in the methods of the present invention. Additionally, the present invention includes local (i.e. intramuscular, intraglandular, subcutaneous, and intracranial) administration methods wherein two or more neurotoxins, such as two or more botulinum toxins, are administered concurrently or consecutively via implant. For example, botulinum toxin type A can be administered via implant until a loss of clinical response or neutralizing antibodies develop, followed by administration via implant of a botulinum toxin type B or E. Alternately, a combination of any two or more of the botulinum serotypes A-G can be locally administered to control the onset and duration of the desired therapeutic result. Furthermore, non-neurotoxin compounds can be administered prior to, concurrently with or subsequent to administration of the neurotoxin via implant so as to provide an adjunct effect such as enhanced or a more rapid onset of denervation before the neurotoxin, such as a botulinum toxin, begins to exert its therapeutic effect.

Detailed Description Text (32):

The present invention also includes within its scope the use of a neurotoxin, such as a botulinum toxin, in the preparation of a medicament, such as a controlled release implant, for the treatment of a movement disorder, and/or a disorder influenced by cholinergic innervation, by local administration via the implant of the neurotoxin.

Other Reference Publication (1):

A. Carruthers, et al., Toxins 99, New Information About the Botulinum Neurotoxins; Dermatol Surg 2000; 26(3): pp. 174-176.

CLAIMS:

1. A pulsatile release botulinum toxin delivery system, comprising:

(a) a carrier;

(b) a botulinum toxin associated with the carrier, thereby forming a pulsatile release botulinum toxin delivery system,

wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a plurality of pulses in vivo upon subdermal implantation of the delivery system in a human patient without a significant immune system response.

3. The delivery system of claim 1, wherein substantial amounts of the botulinum toxin has not be transformed into a botulinum toxoid prior to association of the botulinum toxin with the carrier.

4. The delivery system of claim 1, wherein significant amounts of the botulinum toxin associated with the carrier have a toxicity which is unchanged relative to the toxicity of the botulinum toxin prior to association of the botulinum toxin with the carrier.

6. The delivery system of claim 1, wherein the botulinum toxin can be released from the carrier over of a period of time of from about 10 days to about 6 years.

8. The delivery system of claim 1, wherein the botulinum toxin is selected from the group consisting of botulinum toxin types A, B, C.sub.1, D, E, F and G.

9. The delivery system of claim 1, wherein the botulinum toxin is a botulinum toxin type A.

10. The delivery system of claim 1, wherein the quantity of the botulinum toxin associated with the carrier is between about 1 unit and about 50,000 units of the botulinum toxin.

11. The delivery system of claim 1, wherein the quantity of the botulinum toxin is between about 10 units and about 2,000 units of a botulinum toxin type A.

12. The delivery system of claim 1, wherein the quantity of the botulinum toxin is between about 100 units and about 30,000 units of a botulinum toxin type B.

13. A controlled release system, comprising:

(a) a biodegradable polymer;

(b) between about 10 units and about 100,000 units of a botulinum toxin encapsulated by the polymer carrier, thereby forming a controlled release system, wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a pulsatile manner in vivo upon subdermal implantation of the controlled release system in a human patient over a prolonged period of time extending from about 2 months to about 5 years without a significant immune system response.

14. A method for making a controlled release system, the method comprising the steps of:

(a) dissolving a polymer in a solvent to form a polymer solution;

(b) mixing or dispersing a botulinum toxin in the polymer solution to form a polymer-botulinum toxin mixture, and;

(c) allowing the polymer-botulinum toxin mixture to set or cure, thereby making a

controlled release system for pulsatile release of the botulinum toxin without a significant immune system response.

16. A method for using a pulsatile drug delivery system, the method comprising injection or implantation of a controlled release system which includes a polymeric matrix and a botulinum toxin, thereby treating a movement disorder or a disorder influenced by cholinergic innervation by local administration of a botulinum toxin without a significant immune system response.

17. A pulsatile release botulinum toxin delivery system, comprising:

(a) a carrier comprising a polymer selected from the group of polymers consisting of polylactides, polyglycolides and polyanhydrides, wherein the carrier further comprises a plurality of discrete sets of polymeric, botulinum toxin incorporating microspheres, each set of polymers having a different polymeric composition;

(b) a stabilized botulinum toxin associated with the carrier, thereby forming a pulsatile release botulinum toxin delivery system,

wherein the botulinum toxin comprises:

(a) a first element comprising a binding element able to specifically bind to a neuronal cell surface receptor under physiological conditions,

(b) a second element comprising a translocation element able to facilitate the transfer of a polypeptide across a neuronal cell membrane, and

(c) a third element comprising a therapeutic element able, when present in the cytoplasm of a neuron, to inhibit exocytosis of acetylcholine from the neuron, wherein the therapeutic element can cleave a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), thereby inhibiting the exocytosis of acetylcholine from the neuron, and,

wherein therapeutic amounts of the botulinum toxin can be released from the carrier in a plurality of pulses in vivo upon subdermal implantation of the delivery system in a human patient without a significant immune system response.

18. The delivery system of claim 17, wherein the carrier comprises a plurality of discrete sets of polymeric, botulinum toxin incorporating microspheres, wherein each set of polymers has a different polymeric composition.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Abstract	Claims	KWIC	Draw D
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☐ 10. Document ID: US 6197755 B1

L5: Entry 10 of 19

File: USPT

Mar 6, 2001

DOCUMENT-IDENTIFIER: US 6197755 B1

TITLE: Compositions and methods for delivery of genetic material

Detailed Description Text (75):

Collagenase (clostridiopeptidase A) is a product of clostridium histolyticum. It is a proteolytic enzyme which breaks down native undenatured collagen at physiological

pH and temperature. Collagen comprises about 75% of the dry weight of skin. Collagenase may be prepared according to the procedure of Keller et al., 1963 Arch. Biochem. Biophys. 101:81, which is incorporated herein by reference. It is available commercially as an ointment containing 250 units/gram (Santyl.RTM., Knoll). Preparations may be formulated for parenteral administration containing 1.0 to 1000 units collagenase per mL in a pharmaceutically acceptable carrier, e.g., sterile water for injection, sodium chloride injection, or another pharmaceutically acceptable aqueous injection fluid. Other doses and concentrations, e.g., 5.0 to 500 500 units, preferably 10 to 100 units, which achieve the desired facilitation of the the effect of the genetic construct may be used. For this application collagenase is is injected into the site of administration of the genetic construct, either before, before, after, and/or simultaneously, preferably simultaneously, with the administration of the genetic construct.

#### Detailed Description Paragraph Table (4):

TABLE 2 Bacterial pathogens Pathogenic gram-positive cocci include: pneumococcal; staphylococcal; and streptococcal. Pathogenic gram-negative cocci include: meningococcal; and gonococcal. Pathogenic enteric gram-negative bacilli include: enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigellosis; hemophilus; chancroid; brucellosis; tularemia; yersinia (pasteurella); streptobacillus moniliformis and spirillum; listeria monocytogenes; erysipelotheix rhusiopathiae; diphtheria; cholera; anthrax; donovanosis (granuloma inguinale); and bartonellosis. Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include: syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis. Rickettsial infections include rickettsial and rickettsioses. Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lympho- granuloma venereum; psittacosis; and perinatal chlamydial infections. Pathogenic eukaryotes Pathogenic protozoans and helminths and infections thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

#### CLAIMS:

15. The method of claim 13 wherein said protein is selected from the group consisting of: protein products of oncogenes, myb, myc, fyn, ras, sarc, neu and trk; protein products of translocation gene bcl/abl; p53; EGRF; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Attachment	Claims	KWC	Draw. D
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☐ 11. Document ID: US 6100056 A

L5: Entry 11 of 19

File: USPT

Aug 8, 2000

DOCUMENT-IDENTIFIER: US 6100056 A

TITLE: Nisins

Brief Summary Text (3):

Nisin is a highly modified peptide antibiotic produced, for example, by certain strains of *Lactococcus lactis*. It is of great interest to the food industry because of its efficient antimicrobial activity against a wide range of gram-positive organisms including many spoilage bacteria and food pathogens, for example, *Listeria*, *Clostridia* and *Bacillus* species (12,25).

## CLAIMS:

1. An organism which does not secrete a natural nisA nisin, but which expresses genes for nisin modification, immunity, and translocation out of the cell, wherein the organism is a lactococcal strain.
4. A method of producing the organism of claim 1 comprising selecting a nisin producing organism which contains a nisA gene and insertionally inactivating the nisA gene and restoring the activity of the genes for nisin modification, immunity, and translocation out of the cell.
5. The method according to claim 4 wherein the restoration of the activity of the genes for nisin modification, immunity, and translocation out of the cell is achieved by selection in media containing nisin.
13. An organism which is a *Lactococcus lactis* which does not secrete its natural nisA nisin, but which expresses genes for nisin modification, immunity, and translocation out of the cell.
16. A method of producing the organism of claim 13 comprising selecting a nisin producing organism which contains a nisA gene and insertionally inactivating the nisA gene and restoring the activity of the genes for nisin modification, immunity and translocation out of the cell.
17. The method according to claim 16 wherein the restoration of the activity of the genes for nisin modification, immunity and translocation out of the cell is achieved by selection in media containing nisin.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Examinations	Amendments	Claims	KWIC	Drawings
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☐ 12. Document ID: US 6022950 A

L5: Entry 12 of 19

File: USPT

Feb 8, 2000

DOCUMENT-IDENTIFIER: US 6022950 A

**\*\* See image for Certificate of Correction \*\***

TITLE: Hybrid molecules having translocation region and cell-binding region

Detailed Description Text (57):

The translocation function of the hybrid molecule may be supplied by an appropriate piece of a polypeptide other than diphtheria toxin, but which is capable of translocating in a manner analogous to that of diphtheria toxin (e.g., *Pseudomonas* exotoxin A, botulinum neurotoxin, or ricin), or in any other manner which accomplishes the objective of translocating the functional "third part" of the

hybrid molecule into the cell's cytoplasm.

CLAIMS:

1. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding ligand effective to cause said hybrid molecule to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a chemical entity to be introduced into the cell, wherein each of said first part and said third part is non-native with respect to said naturally occurring protein, and further wherein said covalent bond connecting said second part and said third part is a cleavable bond, provided that when said second part comprises a portion of a translocation domain of Pseudomonas exotoxin, said third part is not a polypeptide.

7. The hybrid molecule of claim 1, wherein said naturally occurring protein of (b) is botulinum neurotoxin.

34. A hybrid molecule comprising a first part, a second part and a third part connected by covalent bonds,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of the translocation domain of diphtheria toxin which translocates said third part across the cytoplasmic membrane and into the cytosol of the cell; and

(c) wherein said third part comprises a chemical entity to be introduced into the cell, wherein said chemical entity and said first part are non-native with respect to said diphtheria toxin, and further wherein said covalent bond connecting said second part and said third part is a cleavable bond.

49. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of Shiga-like toxin which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises an enzymatically active domain of Shiga-like toxin.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Abstract	Claims	KWC	Draw De
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☐ 13. Document ID: US 5981505 A

L5: Entry 13 of 19

File: USPT

Nov 9, 1999

DOCUMENT-IDENTIFIER: US 5981505 A

**\*\* See image for Certificate of Correction \*\***

TITLE: Compositions and methods for delivery of genetic material

Detailed Description Paragraph Table (4):

## TABLE 2

Bacterial pathogens Pathogenic gram-positive cocci include: pneumococcal; staphylococcal; and streptococcal. Pathogenic gram-negative cocci include: meningococcal; and gonococcal. Pathogenic enteric gram-negative bacilli include: enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigellosis; hemophilus; chancroid; brucellosis; tularemia; yersinia (pasteurella); streptobacillus moniliformis and spirillum; listeria monocytogenes; erysipelotheix rhusiopathiae; diphtheria; cholera; anthrax; donovanosis (granuloma inguinale); and bartonellosis. Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include: syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis. Rickettsial infections include rickettsial and rickettsioses. Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections. Pathogenic eukaryotes Pathogenic protozoans and helminths and infections thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

## CLAIMS:

57. The method of claim 51 wherein said hyperproliferative disease-associated protein is selected from the group consisting of: protein products of oncogenes myb, myc, fyn, ras, src, neu and trk; protein products of translocation gene bcr/abl; P53; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Attachments	Claims	KMC	Draw. De
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☐ 14. Document ID: US 5965406 A

L5: Entry 14 of 19

File: USPT

Oct 12, 1999

DOCUMENT-IDENTIFIER: US 5965406 A

**\*\* See image for Certificate of Correction \*\***

TITLE: Recombinant DNAs encoding three-part hybrid proteins

Abstract Text (3):

(b) wherein said second part comprises a portion of a translocation domain of naturally occurring protein selected from the group consisting of diphtheria toxin, botulinum neurotoxin, ricin, cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, pertussis toxin and tetanus toxin, which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

Detailed Description Text (57):

The translocation function of the hybrid molecule may be supplied by an appropriate piece of a polypeptide other than diphtheria toxin, but which is capable of translocating in a manner analogous to that of diphtheria toxin (e.g., *Pseudomonas* exotoxin A, botulinum neurotoxin, or ricin), or in any other manner which accomplishes the objective of translocating the functional "third part" of the hybrid molecule into the cell's cytoplasm.

CLAIMS:

1. A recombinant DNA molecule encoding a hybrid protein comprising a first part, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein selected from the group consisting of diphtheria toxin, botulinum neurotoxin, ricin, cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, pertussis toxin and tetanus toxin, which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said third part is non-native with respect to said naturally occurring protein of (b).

29. A recombinant DNA molecule encoding a hybrid protein comprising a first part, a second part and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of the translocation domain of diphtheria toxin which translocates said third part across the cytoplasmic membrane and into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said polypeptide entity is non-native with respect to said diphtheria toxin.

45. A method of preparing a hybrid protein comprising a first part, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein selected from the group consisting of diphtheria toxin, botulinum neurotoxin, ricin, cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, pertussis toxin and tetanus toxin, which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said third part is non-native with respect to said naturally occurring protein of (b) comprising the steps of:

providing a cell transformed with a recombinant DNA molecule encoding the hybrid protein, and

culturing the transformed cell to allow expression of the recombinant DNA molecule such that the hybrid protein is produced.

48. A method of preparing a hybrid protein comprising a first part, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause the hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of diphtheria toxin which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said third part is non-native with respect to said diphtheria toxin, comprising the steps of:

providing a cell transformed with a recombinant DNA molecule encoding the hybrid protein, and

culturing the transformed cell to allow expression of the recombinant DNA molecule such that the hybrid protein is produced.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Experiments	Figures	Claims	KWIC	Draw D
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15. Document ID: US 5962428 A

L5: Entry 15 of 19

File: USPT

Oct 5, 1999

DOCUMENT-IDENTIFIER: US 5962428 A

TITLE: Compositions and methods for delivery of genetic material

#### Detailed Description Text (74):

Collagenase (clostridiopeptidase A) is a product of clostridium histolyticum. It is a proteolytic enzyme which breaks down native undenatured collagen at physiological pH and temperature. Collagen comprises about 75% of the dry weight of skin. Collagenase may be prepared according to the procedure of Keller et al., 1963 Arch. Biochem. Biophys. 101:81, which is incorporated herein by reference. It is available commercially as an ointment containing 250 units/gram (Santyl.RTM.,

Knoll). Preparations may be formulated for parenteral administration containing 1.0 to 1000 units collagenase per mL in a pharmaceutically acceptable carrier, e.g., sterile water for injection, sodium chloride injection, or another pharmaceutically acceptable aqueous injection fluid. Other doses and concentrations, e.g., 5.0 to 500 500 units, preferably 10 to 100 units, which achieve the desired facilitation of the the effect of the genetic construct may be used. For this application collagenase is is injected into the site of administration of the genetic construct, either before, before, after, and/or simultaneously, preferably simultaneously, with the administration of the genetic construct.

Detailed Description Paragraph Table (4):

TABLE 2 Bacterial pathogens Pathogenic gram-positive cocci include: pneumococcal; staphylococcal; and streptococcal. Pathogenic gram-negative cocci include: meningococcal; and gonococcal. Pathogenic enteric gram-negative bacilli include: enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigellosis; hemophilus; chancroid; brucellosis; tularemia; yersinia (pasteurella); streptobacillus moniliformis and spirillum; listeria monocytogenes; erysipelotheix rhusiopathiae; diphtheria; cholera; anthrax; donovanosis (granuloma inguinale); and bartonellosis. Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include: syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis Rickettsial infections include rickettsial and rickettsioses. Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections. Pathogenic eukaryotes Pathogenic protozoans and helminths and infections thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

CLAIMS:

26. The method of claim 20 wherein said DNA molecule comprises a DNA sequence that encodes a protein selected from the group consisting of: protein products of oncogenes myb, myc, fyn, ras, sarc, neu and trk; protein products of translocation gene bcl/abl; p53; EGRF; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Abstract	Claims	KWIC	Draw D
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☐ 16. Document ID: US 5817637 A

L5: Entry 16 of 19

File: USPT

Oct 6, 1998

DOCUMENT-IDENTIFIER: US 5817637 A

TITLE: Genetic immunization

Detailed Description Paragraph Table (4):

TABLE 2 Bacterial pathogens Pathogenic gram-positive cocci include: pneumococcal; staphylococcal; and streptococcal. Pathogenic gram-negative cocci include: meningococcal; and gonococcal. Pathogenic enteric gram-negative bacilli include: enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigellosis; hemophilus; chancroid; brucellosis; brucellosis; tularemia; yersinia (pasteurella); streptobacillus moniliformis and spirillum; listeria monocytogenes; erysipelotheix rhusiopathiae; diphtheria; cholera; anthrax; donovanosis (granuloma inguinale); and bartonellosis. Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include: syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis. Rickettsial infections include rickettsial and rickettsioses. Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections. Pathogenic eukaryotes Pathogenic protozoans and helminths and infections thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

## CLAIMS:

26. The method of claim 25 wherein said hyperproliferative disease-associated protein is selected from the group consisting of: protein products of oncogenes myb, myc, fyn, ras, src, neu and trk; protein products of translocation gene bcr/abl; P53; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstract	Abstract	Claims	KMC	Draw De
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☐ 17. Document ID: US 5763190 A

L5: Entry 17 of 19

File: USPT

Jun 9, 1998

DOCUMENT-IDENTIFIER: US 5763190 A

TITLE: Methods for the identification of compounds capable of inducing the nuclear translocation of a receptor complex comprising the glucocorticoid receptor type II and viral protein R interacting protein

Brief Summary Text (75):

The conjugated compositions and fusion proteins of the present invention may be used to deliver active agents to the nucleus of cells in both in vitro and in vivo protocols. As discussed above, active agents include nucleic acid molecules, drugs, radioisotopes, and toxins for example. In some embodiments, the active agent is selected from the group consisting of: methotrexate, doxorubicin, daunorubicin, cytosinarabioside, etoposide, 5-4 fluorouracil, melphalan, chlorambucil, cis-platinum, vindesine, mitomycin, bleomycin, purothionin, macromomycin, 1,4-benzoquinone derivatives, trenimon, ricin, ricin A chain, Pseudomonas exotoxin, diphtheria toxin, Clostridium perfringens phospholipase C, bovine pancreatic

ribonuclease, pokeweed antiviral protein, abrin, abrin A chain, cobra venom factor, gelonin, saporin, modeccin, viscumin, volkensin, alkaline phosphatase, nitroimidazole, metronidazole and misonidazole. Such active agents are conjugated to Vpr or fragments of Vpr that bind to Rip-1 and induce translocation of Rip-1 to the nucleus. Vpr may be isolated from natural sources or produced by a variety of well known means such as those described in U.S. Ser. No. 08/019,601 filed Feb. 19, 1993 and U.S. Ser. No. 08/167,608 filed Dec. 15, 1993, both of which are incorporated herein by reference. Fragments of Vpr that bind to Rip-1 and induce translocation of Rip-1 to the nucleus are fragments of Vpr as described herein, particularly Example 3, and may be identified as described herein.

## CLAIMS:

1. An in vitro method of identifying compounds that induce glucocorticoid receptor type II (GR-II) and viral protein R interacting protein (Rip-1) complex translocation comprising the following steps:

- a) preparing cells expressing Rip-1 and the GR-II wherein Rip-1 and GR-II are capable of forming a cytosolic Rip-1/GR-II receptor complex;
- b) contacting said cells with a test compound capable of inducing Rip-1/GR-II receptor complex cytoplasmic to nuclear translocation;
- c) detecting the level of Rip-1/GR-II receptor cytoplasmic to nuclear translocation in said cells in the presence of the test compound; and,
- d) performing a control assay that detects the level of Rip-1/GR-II receptor complex nuclear translocation in the absence of said test compound;

wherein detection of a higher level of cytoplasmic to nuclear translocation of the Rip-1/GR-II receptor complex is indicative of said compound being capable of inducing Rip-1/GR-II receptor complex translocation.

2. The method of claim 1 wherein Rip-1/GR-II receptor complex nuclear translocation is detected through the subcellular fractionation of said cells into soluble and insoluble antigen-containing fractions, followed by the addition of Rip-1-specific antibodies to each fraction.

5. An in vitro method of inducing glucocorticoid receptor type II (GR-II) and viral protein R interacting protein (Rip-1) complex nuclear translocation in cells comprising the following steps:

- a) preparing cells expressing Rip-1 and GR-II wherein said proteins are capable of forming a cytosolic Rip-1/GR-II receptor complex; and,
- b) contacting said cells with the human immunodeficiency virus type 1 (HIV-1) Vpr protein under conditions wherein said Vpr protein binds to the cytosolic Rip-1/GR-II receptor complex thereby generating a Vpr/Rip-1/GR-II complex that subsequently undergoes cytoplasmic to nuclear translocation.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Abstracts	Abstracts	Claims	KWIC	Draw. De
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☐ 18. Document ID: US 5677274 A

L5: Entry 18 of 19

File: USPT

Oct 14, 1997

DOCUMENT-IDENTIFIER: US 5677274 A

**\*\* See image for Certificate of Correction \*\***

TITLE: Anthrax toxin fusion proteins and related methods

Detailed Description Text (54):

An intracellular pathogen specific protease site can be introduced into any natural or recombinant toxin for which proteolytic cleavage is required for toxicity. For example, one can replace the anthrax PA trypsin cleavage site (R164-167) of PA with the HIV-1 protease site. Alternatively, the diphtheria toxin disulfide loop sequence (see O'Hare, et al. FEBS 273 (1, 2): 200-204 (October 1990)) can be replaced with the HIV-1 protease cleavage site in order to obtain a toxin specific to HIV-1 infected cells. Similarly, the normally occurring diphtheria toxin sequence at residues 191-194 (Williams, et al. J. Biol. Chem. 265(33): 20673-20677 (1990)) can be replaced by an intracellular pathogen specific protease site such as the HIV-1 protease cleavage sequence. The DAB486-IL-2 fusion toxin of Williams and the improved DAB389-IL-2 toxin are effective on HIV-1 infected cells, which express high levels of the IL-2 receptor. Williams, J. Biol. Chem. 265:20673. Addition of the HIV-1 protease cleavage site would provide a further degree of specificity. Similarly, the botulinum toxin C2 toxin is like the anthrax toxin in requiring a cleavage within a native protein subunit (see Ohishi and Yanagimoto, Infection and Immunity 60(11): 4648-4655 (November 1992)), so it too can be made specific for cells infected by an intracellular pathogen such as HIV-1.

Other Reference Publication (17):

Ohishi, I., et al., "Visualizations of Binding and Internalization of Two Nonlinked Protein Components of Botulinum C.sub.2 Toxin in Tissue Culture Cells," Infection and Immunity, 60(11):4648-4655 (Nov. 1992).

## CLAIMS:

1. A method for targeting compounds having a desired biological activity not present on native anthrax lethal factor (LF) to a specific cell population, comprising:

a) administering to the cell population a first compound comprising a first protein consisting essentially of:

i) the translocation domain and the anthrax lethal factor (LF) binding domain of the the native anthrax protective antigen (PA) protein, and

ii) a ligand domain that specifically binds the first protein to a target on the surface of the cell population to bind the first compound to said surface; and

b) administering to the resultant cell population a second compound comprising a fusion protein or conjugate consisting essentially of:

i) the anthrax protective antigen (PA) binding domain of the native anthrax lethal factor (LF) protein, chemically attached to

ii) a biological activity-inducing polypeptide to bind the second compound to the first compound on the surface of the cell population, internalize the second compound into the cell population, and effect the activity of the polypeptide therein.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Abstracts	Claims	KWIC	Draw. De
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L5: Entry 19 of 19

File: USPT

Jan 14, 1997

DOCUMENT-IDENTIFIER: US 5593972 A

TITLE: Genetic immunization

Detailed Description Text (435):

Pathogenic anaerobic bacteria include: tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include: syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include: actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis.

## CLAIMS:

7. The method of claim 6 wherein said DNA molecule comprises a DNA sequence encoding a target protein selected from the group consisting of: protein products of oncogenes myb, myc, fyn, ras, src, neu and trk; protein products of translocation gene bcr/abl; P53; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.

Full	Title	Citation	Front	Review	Classification	Date	Reference	Sequence	Alignment	Claims	KWIC	Draw. De
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Clear	Generate Collection	Print	Fwd Refs	Bkwd Refs
Generate OACS				

Terms	Documents
L4 and (botulin or botulinum or botoxin or clostridia or clostridium or clostridial or neurotoxin or neuro-toxin)	19

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be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *Clostridium* species are contemplated as immunogens. Examples of these toxins include the neuraminidase toxin of *C. butyricum*, *C. sordellii* toxins HT and LT, toxins A, B, C, D, E, F, and G of *C. botulinum* and the numerous *C.*

5 *perfringens* toxins. In one preferred embodiment, toxins A, B, and E of *C. botulinum* are contemplated as immunogens. Table 2 above lists various *Clostridium* species, their toxins and some antigens associated with disease.

TABLE 2  
Clostridial Toxins

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Organism	Toxins and Disease-Associated Antigens
<i>C. botulinum</i>	A, B, C <sub>1</sub> , C <sub>2</sub> , D, E, F, G
<i>C. butyricum</i>	Neuraminidase
<i>C. difficile</i>	A, B, Enterotoxin (not A nor B), Motility Altering Factor, Low Molecular Weight Toxin, Others
<i>C. perfringens</i>	$\alpha$ , $\beta$ , $\epsilon$ , $\iota$ , $\gamma$ , $\delta$ , $\nu$ , $\theta$ , $\kappa$ , $\lambda$ , $\mu$ , $\upsilon$
15 <i>C. sordellii</i> <i>C. bifermentans</i>	HT, LT, $\alpha$ , $\beta$ , $\gamma$
<i>C. novyi</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$ , $\epsilon$ , $\zeta$ , $\nu$ , $\theta$
<i>C. septicum</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$
<i>C. histolyticum</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$ , $\epsilon$ plus additional enzymes
20 <i>C. chauvoei</i>	$\alpha$ , $\beta$ , $\gamma$ , $\delta$

It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin (e.g., *C. perfringens* type A enterotoxin) may be used as an effective therapeutic against one or more toxin(s) produced by other members of the genus *Clostridium* or other toxin producing organisms (e.g., *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus*

25 *mutans*, *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa*, other *Pseudomonas*

for a number of *C. botulinum* toxins isolated from different strains within a given serotype. The *C. botulinum* toxins contain about 1250-1300 amino acid residues. On the DNA level, the overall degree of homology between *C. botulinum* serotypes A, B, C, D and E toxins averages between 50 and 60% identity with a greater degree of homology being found between H chain-encoding regions than between those encoding L chains [Whelan *et al.* (1992) Appl. Environ. Microbiol. 58:2345]. The degree of identity between *C. botulinum* toxins on the amino acid level reflects the level of DNA sequence homology. The most divergent area of DNA and amino acid sequence is found within the carboxy-terminal area of the various *C. botulinum* H chain genes. This portion of the toxin (*i.e.*, H<sub>C</sub> or the C fragment) plays a major role in cell binding. As toxin from different serotypes is thought to bind to distinct cell receptor molecules, it is not surprising that the toxins diverge significantly over this region.

Within a given serotype, small variations in the primary amino acid sequence of the botulinal toxins isolated from different strains has been reported [Whelan *et al.* (1992), *supra* and Minton (1995), *supra*]. The present invention contemplates fusion proteins comprising portions of *C. botulinum* toxins from serotypes A-G including the variants found among different strains within a given serotype. The present invention provides oligonucleotide primers which may be used to amplify the C fragment or receptor-binding region of the toxin gene from various strains of *C. botulinum* serotype A, serotype B, serotype C (C1 and C2), serotype D, serotype E, serotype F and serotype G. A large number of different strains of *C. botulinum* serotype A, serotype B, serotype C, serotype D serotype E and serotype F are available from the American Type Culture Collection (ATCC; Rockville, MD). For example, the ATCC provides the following: Type A strains: 174 (ATCC 3502), 457 (ATCC 17862), and NCTC 7272 (ATCC 19397); Type B strains: 34 (ATCC 439), 62A (ATCC 7948), NCA 213 B (ATCC 7949), 13114 (ATCC 8083), 3137 (ATCC 17780), 1347 (ATCC 17841), 2017 (ATCC 17843), 2217 (ATCC 17844), 2254 (ATCC 17845) and VP 1731 (ATCC 25765); Type C strains: 2220 (ATCC 17782), 2239 (ATCC 17783), 2223 (ATCC 17784; a type C- $\beta$  strain; C- $\beta$  strains produce C2 toxin), 662 (ATCC 17849; a type C-